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Vaginal microecological disorder and gynecological diseases

Edited by
Zhangran Chen, Lei Cheng, Qinping Liao,
Tingtao Chen and Ruonan Wu

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Vaginal microecological disorder and gynecological diseases

Topic editors

Zhangran Chen — Shenzhen Wedge Microbiology Research Co.LTD, China
Lei Cheng — Biogas Institute of Ministry of Agriculture, Chinese Academy of Agricultural Sciences, China
Qinping Liao — Tsinghua University, China
Tingtao Chen — Nanchang University, China
Ruonan Wu — Pacific Northwest National Laboratory (DOE), United States

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EDITED BY
Nahed Ismail,
University of Illinois Chicago, United States

REVIEWED BY
Eva Pericolini,
University of Modena and Reggio Emilia, Italy

*CORRESPONDENCE
Zhangran Chen
zhangran22105@163.com
Qinping Liao
qinpingliao@sina.com

†These authors have contributed equally to this work

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Editorial: Vaginal microecological disorder and gynecological diseases

Tingtao Chen[1†], Ruonan Wu[2†], Lei Cheng[3†], Qinping Liao[4*] and Zhangran Chen[5*]

[1]National Engineering Research Center for Bioengineering Drugs and the Technologies, Institute of Translational Medicine, Nanchang University, Nanchang, China, [2]Biological Science Division, Pacific Northwest National Laboratory, Richland, WA, United States, [3]Key Laboratory of Development and Application of Rural Renewable Energy, Biogas Institute of Ministry of Agriculture and Rural Affairs, Chengdu, China, [4]Department of Obstetrics and Gynecology, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing, China, [5]Shenzhen Wedge Microbiology Research Co.Ltd, Shenzhen, China

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Editorial on the Research Topic
Vaginal microecological disorder and gynecological diseases

Researchers have been increasingly interested in investigating microbial communities in the lower and upper reproductive tract and their impact on female reproductive health. A healthy vaginal ecosystem is dominated by *Lactobacillus* spp., which can be infected by various pathogens, including human papillomavirus (HPV) and human immunodeficiency virus, and is susceptible to dysbiosis of its microbiota. Pathogenic overgrowth results in the development of diseases, including aerobic vaginitis, bacterial vaginosis(BV), cytolytic vaginosis, trichomonas vaginitis, vulvovaginal candidiasis (VVC), urinary tract infections, sexually transmitted infections (STIs), and gynecological oncology. Determining the associations between the vaginal microbiota and these diseases can provide valuable insights for detecting diagnosing, and interventing complex female illnesses.

This Research Topic comprises fifteen papers, consisting of three subtopics: vaginal inflammation, HPV, and reproductive health.

The first subtopic on vaginal inflammation, comprises one review, two BV-related research articles, and two VVC-related articles. Gao et al. observed the top five potential common pathogens of vaginal infection, with *Haemophilus influenzae* having the highest prevalence, followed by *Streptococcus pyogenes*, *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus*. Zhou et al. examined the vaginal microbiota of patients with BV before and after antibiotic treatment, compared with healthy controls and identified *Lactobacillus iners* as a potential predictive indicator of clinical outcomes in patients with BV. Shen et al. showed that a postbiotic gel alleviated BV symptoms by increasing *Lactobacillus* spp., and reducing the presence of potential vaginal pathogens. Sun et al. summarized the changes in vaginal microbiota during VVC infection and highlighted the potential use of *Lactobacillus* spp. as probiotics for VVC treatment. Considering the high costs and potential side effects associated with antifungal agents for VVC treatment, Lu et al.

found that X33 antimicrobial oligopeptide (X33 AMOP) effectively inhibited the virulence of *C.albicans* by reducing phospholipase activity and disrupting mycelium formation. Notably, while *Lactobacillus* spp. has shown promise in preventing and treating vaginal inflammation, clinical data regarding its efficacy remain limited and require further exploration.

Four papers discuss the subtopic of HPV and its relationship with cervical cancer. A et al. discovered a higher prevalence of vaginal infections and cervical STIs in the HPV-positive group than in the HPV-negative group. To explore the impact of probiotics on HPV persistence and clearance, Zeng et al. compared 90 patients with HPV and 45 healthy individuals and found that probiotics, as an interferon adjuvant therapy, effectively enhanced virus clearance in some patients. Patients with HPV clearance had significantly lower alpha diversity, accompanied by a decreased abundance of *Fusobacterium*, *Bacteroides*, *Neisseria* and *Helicobacter*, than those in the HPV-persistent group. Zhu et al. observed negative correlation between interleukin-2 (IL-2) levels and the risk of cervical intraepithelial neoplasia in Chinese women, regardless of high-risk HPV infection. Furthermore, Li et al. found that vaginal microecological abnormalities might contribute to a higher false-positive diagnosis rate of atypical squamous cells of undetermined significance, which is diagnosed as precancerous lesions. These findings highlight the significant impact of vaginal microbiota on female health, emphasizing the importance of understanding and maintaining a balanced microbiome.

Six papers were included in the last subtopic of reproductive health. Chao et al. collected uterine lavage samples via hysteroscopy from women with endometrial hyperplasia (EH) or endometrial cancer (EC) and found an increased relative abundance of two plastic-degrading bacteria, *Bacillus pseudofirmus*, and *Stenotrophomonas rhizophila*, in the endometrial lavage microbiota of women with EC/EH. Liang et al. analyzed the uterine cavity, cervix, and vagina samples of 134 patients with infertility and revealed that endometrial microbiota composed of *Staphylococcus*, *Gardnerella*, *Atobor*, *Streptococcus, Peptostreptococcus*, *Chlamydia*, *Fusobacterium* and *Acinetobacter* are related to CE and EP. Wang et al. discovered that vaginal bacteria, including *Ensifer*, *Devosia*, *Bosea*, *Cellomonas*, *Helicobacter*, and *Sphingopyxis*, as well as specific endometrial microbiota, including *Candidatus Symbiobacter*, *Odoribacter*, *Blautia*, *Nocardioides*, and *Ileibacterium*, exhibited predictive value for embryo arrest. Xie et al. found that vaginal microbiota transfer to newborns could help restore the disturbed microbiome caused by cesarean section delivery, resulting in a microbial composition similar to that of infants born through vaginal delivery. Dong et al. focused on recent advancements in understanding the interactions between microbiota and the cervical mucosal barrier. Furthermore, they found a significant impact of host-microbiota interactions on STIs outcomes. For instance, *Chlamydia trachomatis* infection, can cause tubal inflammation, fibrosis, and even obstruction, which have adverse effects on pregnancy. Tian et al. found that oral antibiotics can induce gut dysbiosis in DBA2/J mice, contributing to the development of *Chlamydia*-induced hydrosalpinx in the upper genital tract.

We reviewed recent research efforts that could further guide the discovery of the underlying mechanisms of microbial-mediated vaginal diseases and provide readers with valuable insights into the prediction, prevention, and treatment of gynecological diseases through reproductive tract microorganisms. In the future, more rigorous and clinically focused research is necessary to explore the specific mechanisms by which microorganisms contribute to the occurrence and development of female diseases. A deeper understanding of these mechanisms will provide crucial information for the diagnosis, treatment strategies, and development and optimization of drugs, probiotics, postbiotics, and vaginal microbiota transplantation.

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Conflict of interest

ZC is employed by Shenzhen Wedge Microbiology Research Co. Ltd.

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Alessandra Fusco,
Universitàdella Campania Luigi Vanvitelli, Italy
Michael Bording-Jorgensen,
University of Alberta, Canada

*CORRESPONDENCE
Kai-Yun Wu
m7030@cgmh.org.tw

†These authors have contributed equally to this work and share first authorship

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Analysis of endometrial lavage microbiota reveals an increased relative abundance of the plastic-degrading bacteria *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* in women with endometrial cancer/endometrial hyperplasia

Angel Chao[1,2†], An-Shine Chao[1,3†], Chiao-Yun Lin[1,2], Cindy Hsuan Weng[1,2], Ren-Chin Wu[2,3], Yuan-Ming Yeh[4], Shih-Sin Huang[1,2], Yun-Shien Lee[5,6], Chyong-Huey Lai[1,2], Huei-Jean Huang[1,2], Yun-Hsin Tang[1,2], Yu-Shan Lin[1,3], Chin-Jung Wang[1,2] and Kai-Yun Wu[1,2*]

[1]Department of Obstetrics and Gynecology, Linkou Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taoyuan, Taiwan, [2]Gynecologic Cancer Research Center, Linkou Chang Gung Memorial Hospital, Taoyuan, Taiwan, [3]Department of Obstetrics and Gynecology, New Taipei Municipal Tu Cheng Hospital, New Taipei City, Taiwan, [4]Department of Pathology, Linkou Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taoyuan, Taiwan, [5]Genomic Medicine Research Core Laboratory, Chang Gung Memorial Hospital, Taoyuan, Taiwan, [6]Department of Biotechnology, Ming-Chuan University, Taoyuan, Taiwan

The pathogenic influences of uterine bacteria on endometrial carcinogenesis remain unclear. The aim of this pilot study was to compare the microbiota composition of endometrial lavage samples obtained from women with either endometrial hyperplasia (EH) or endometrial cancer (EC) *versus* those with benign uterine conditions. We hypothesized that specific microbiota signatures would distinguish between the two groups, possibly leading to the identification of bacterial species associated with endometrial tumorigenesis. A total of 35 endometrial lavage specimens (EH, n = 18; EC, n = 7; metastatic EC, n = 2; benign endometrial lesions, n = 8) were collected from 32 women who had undergone office hysteroscopy. Microbiota composition was determined by sequencing the V3–V4 region of 16S rRNA genes and results were validated by real-time qPCR in 46 patients with EC/EH and 13 control women. Surprisingly, we found that *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* – two plastic-degrading bacterial species – were over-represented in endometrial lavage specimens collected from patients with EC/EH. Using computational analysis, we found that the functional profile of endometrial microbiota in EC/EH was associated with fatty acid and amino acid metabolism. In summary, our hypothesis-generating

data indicate that the plastic-degrading bacteria *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* are over-represented within the endometrial lavage microbiota of women with EC/EH living in Taiwan. Whether this may be related to plastic pollution deserves further investigation.

KEYWORDS

microbiota, endometrial cancer, endometrial hyperplasia, plastics, environmental pollution

Introduction

Despite decades of intense research, endometrial cancer (EC) remains a substantial public health problem in women (Lortet-Tieulent et al., 2018; Zhang et al., 2019). While the underlying reasons are multifaceted (Felix and Brinton, 2018), growing evidence suggests that environmental pollution can be linked with an increased risk of EC (Dunnick et al., 2015; Mallozzi et al., 2017). Interestingly, exposure to estrogen-mimicking endocrine disruptors – including certain preservatives and industrial plasticizers that can be biologically active at extremely low levels (Hiroi et al., 2004; Chou et al., 2017) – has been related to endometrial carcinogenesis (Yaguchi, 2019; Wen et al., 2020). Furthermore, the ability of plastic particles to accumulate in human tissues, including the reproductive tract, is well established (Zlatnik, 2016; Waring et al., 2018).

Prior studies using molecular techniques have reported that the human endometrium has a resident microbiota dominated by *Lactobacillus*, *Pseudomonas*, and *Acinetobacter* (Chen et al., 2017). In recent years, there have been attempts to investigate the role of uterine dysbiosis in various intrauterine diseases (Baker et al., 2017). Interestingly, plastic pollution has the potential to affect human tissue microbiota (Velmurugan et al., 2017; Lu et al., 2019) either *via* direct toxic effects or by providing supplemental carbon sources (Lear et al., 2021). However, there are limited data available to determine possible links between uterine dysbiosis and endometrial tumorigenesis (Walther-Antonio et al., 2016; Chen et al., 2021; Lu et al., 2021).

Genetic sequencing of uterine lavage samples obtained *via* office hysteroscopy has recently emerged as a less invasive approach for real-time diagnosis and monitoring of EC (Chao et al., 2022). By taking advantage of this technique, the goal of this pilot study was to compare the microbiota composition of endometrial lavage samples obtained from Taiwanese women with either endometrial hyperplasia (EH) or EC *versus* those with benign uterine conditions. We hypothesized that specific microbiota signatures would distinguish between the two groups, possibly leading to the identification of bacterial species associated with endometrial tumorigenesis. Surprisingly, the key dysbiosis identified in endometrial lavage samples collected from women with EC/EH was an increased relative abundance of the two plastic-degrading bacteria *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* (Dela Torre et al., 2018; Wei et al., 2018; Danso et al., 2019; Atanasova et al., 2021).

Materials and methods

Participants

Women with recent abnormal uterine bleeding due to a suspected endometrial lesion (e.g., polyp, myoma, EH, or EC) who were referred for an office hysteroscopy (Salazar and Isaacson, 2018) were eligible, as were those with either a newly diagnosed or known EH/early EC who had undergone fertility-preserving treatment (Chao et al., 2011; Gunderson et al., 2012; Hubbs et al., 2013; Yang et al., 2020). The enrolment started in July 2020 and concluded in June 2021. Women with suspected endometritis and those unwilling to participate were excluded. Endometrial biopsies collected during office hysteroscopy were used for achieving a final pathological diagnosis. Ethical approval was granted by the Institutional Review Board (reference number: 202100083B0) of the Chang Gung Memorial Hospital, Taiwan. All women provided written informed consent before enrolment.

Collection of endometrial lavage specimens

Office hysteroscopy was performed with the vaginoscopic technique (Vitale et al., 2020). Prior to endometrial biopsies, normal saline was instilled to provide distension and irrigation of the uterine cavity. Endometrial lavage samples (25 mL) obtained using a continuous-flow rigid hysteroscopy system (sheath diameter: 4 mm; Richard Wolf GmbH, Knittlingen, Germany) were collected in sterile tubes and centrifuged at 3200 rpm for 20 min at 4°C. Cell pellets were suspended in PBS, washed with a red blood cell lysis solution, incubated at

room temperature for 30 min, and centrifuged at 3000 rpm for 10 min. After removal of the supernatant, pellets were stored -80°C until analysis (Nair et al., 2016).

Bacterial DNA preparation, library construction, and sequencing

Bacterial DNA for microbiota analysis was extracted using a QiaAmp DNA Microbiome Kit (Qiagen, Hilden, Germany). A Qubit dsDNA High Sensitivity Assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the concentration and quality of purified DNA. The protocol for library construction and sequencing has been previously described in detail (Lin et al., 2020). In brief, a 16S rRNA gene amplicon library targeting the 16S rRNA V3–V4 region was initially constructed. Illumina adaptor overhang nucleotide sequences were subsequently added to gene-specific sequences. The 16S rRNA gene amplicon PCR primers were as follows: forward, 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'.

Bioinformatics analysis of amplicon library sequences

The details of bioinformatics analysis of amplicon library sequences have been published (Lin et al., 2020). Briefly, sequencing reads were initially de-multiplexed using the Miseq Reporter tool v2.6 (Illumina, San Diego, CA, USA) based on sample barcodes. The Usearch tool v11 (https://drive5.com/) was used for processing raw reads. A chimera removal step was implemented to ensure that any sequencing-related error was removed through the selection of qualified reads. Species richness and diversity were determined based on the number of bacterial species assigned by amplicon sequence variants (ASVs). We estimated richness using the Observed ASV and Chao1 indices. We also obtained measures of α-diversity – which focuses on variation within a community (e.g., Shannon index and Gini-Simpson index) and β-diversity – which quantifies dissimilarities between communities based on the principal component analysis plot constructed with unweighted UniFrac. Finally, we used LEfSe20 to identify the taxonomy of bacteria that were most likely to distinguish women with either EH or EC *versus* those with benign uterine conditions.

Real time qPCR

The TaqMan gene expression assay (Applied Biosystems, Foster City, CA, USA) was used to quantify DNA from *Bacillus pseudofirmus* (Assay ID: ART2CTZ) and *Stenotrophomonas rhizophila* (Assay ID: APH6GAT). Data were normalized using 16S rRNA expression levels (Assay ID: Ba04930791_s1). When the fluorescent signal did not increase until 40 cycles, the sample was assigned an arbitrary value of 40. The -ΔΔCt was calculated according to the following formula: ΔCt (bacteria) – ΔCt (internal control). Correlation analyses of ΔCt values were performed to assess their reciprocal associations (Lin et al., 2020).

Microbiota data analysis

ASV relative abundances (expressed as percentages) were log-transformed prior to further analyses (Lin et al., 2020). The metagenome content was predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Data concerning the mid-vaginal microbiota of healthy non-pregnant women and the cervical microbiota of pregnant women were obtained from the Human Microbiome Project (HMP) (Human Microbiome Project C, 2012a; Human Microbiome Project C, 2012b) and the Integrative Human Microbiome Project (iHMP) (Integrative H.M.P.R.N.C, 2019), respectively. Statistical analyses were performed using R (http://www.r-project.org/), unless otherwise indicated.

Results

A total of 35 endometrial lavage specimens (EH, n = 18; EC, n = 7; metastatic EC, n = 2; benign endometrial lesions, n = 8) were collected from 32 women who had undergone office hysteroscopy (Supplementary Figure 1; Supplementary Table 1). For the purpose of analysis, samples were categorized as follows: patients with premalignant and malignant endometrial lesions (EC/EH; n = 27) *versus* control women (n = 8).

Richness and diversity of endometrial lavage microbiota

Endometrial lavage bacterial microbiota was characterized using 16S rRNA V3–V4 gene sequence analysis. Patients with EC/EH did not differ from control women in terms of species richness (Observed ASV and Chao1 indices). However, the former group had a higher α-diversity compared with the latter (Shannon index, $P = 0.034$; Gini-Simpson index, $P = 0.044$; Figure 1A). While the two study groups did not differ significantly in terms of taxonomic β-diversity, there was a trend towards a higher concentration in the middle portion for patients with EC/EH (Figure 1B).

Composition of endometrial lavage microbiota

The relative abundance of endometrial lavage bacteria was analyzed at three taxonomic ranks (class, genus, and species). At the class level, *Alphaproteobacteria* were more commonly represented in patients with EC/EH than in control women (P = 0.0307; Figure 2). At the genus level, the *Bacillus* (P = 0.005), *Stenotrophomonas* (P = 0.0157), *Phyllobacterium* (P = 0.0495), *Pseudomonas* (P = 0.0495), *Brevundimonas* (P = 0.0373), and *Rhodococcus* (P = 0.0492) genera were over-represented in patients with EC/EH (Figure 3). Conversely, *Lactobacilli* and *Bifidobacterium* were under-represented. At the species level, we found that *Bacillus pseudofirmus* (P = 0.0018; Figure 4A) and *Stenotrophomonas rhizophila* (P = 0.0288; Figure 4B) were over-represented in patients with EC/EH. This finding was validated using real-time qPCR in a larger cohort of patients with EC/EH (54 lavage samples obtained from 46 patients) and control women (13 lavage samples obtained from 13 women; Supplementary Table 1). The results confirmed the higher relative abundance of both *Bacillus pseudofirmus*(Figure 4C) and *Stenotrophomonas rhizophila* (Figure 4D) in the endometrial lavage microbiota of the former group (Supplementary Table 2).

Linear discriminant analysis of taxonomic profiles

A linear discriminant analysis of effect size (LEfSe) was undertaken to compare the taxonomic profiles of endometrial lavage microbiota in patients with EC/EH *versus* control women (Figure 5A). Significant intergroup differences were observed at different taxonomic ranks (class, family, genus, and species; Figure 5B). This analysis consistently supported the differences in the relative abundance of *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* between the two study groups (Supplementary Table 3).

Functional profile of endometrial lavage microbiota

The PICRUSt tool was implemented to predict the functional profile of endometrial lavage microbiota in patients with EC/EH. The results revealed an involvement of the following two pathways (Figure 6 and Supplementary Table 4): 1) fatty acid metabolism (including fatty acid biosynthesis and biosynthesis of unsaturated fatty acids; and 2) amino acid metabolism (including D-arginine and D-ornithine metabolism; valine, leucine, and isoleucine degradation; lysine degradation; tryptophan metabolism; histidine metabolism; glutathione metabolism; and beta-alanine metabolism).

Validation by comparison between endometrial lavage and vaginal microbiota

Finally, sequencing data were compared using the HMP and iHMP datasets (Figure 7 and Supplementary Table 5). We found a stepwise decrease in the relative abundance of the genus *Lactobacillus* in the following microbiotas: 1) mid-vaginal microbiota of healthy non-pregnant women, 2) cervical microbiota of pregnant women, 3) endometrial lavage microbiota of women with benign endometrial lesions, and 4) endometrial lavage microbiota of women with EC/EH.

Discussion

The novel finding of this pilot, hypothesis-generating study is that *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* – two plastic-degrading bacteria (Dela Torre et al., 2018; Wei et al., 2018; Danso et al., 2019; Atanasova et al., 2021) – are over-represented within the endometrial lavage microbiota of Taiwanese women with EC/EH. On the one hand, *Bacillus pseudofirmus* is a facultative aerobic alkaliphile bacterium characterized by the ability to degrade inert polyethylene-based plastics, including low-density polyethylene (LDPE) (Dela Torre et al., 2018; Atanasova et al., 2021). On the other hand, *Stenotrophomonas rhizophila* is not only an efficient degrader of polyvinyl alcohol (Wei et al., 2018) but can also remove heavy metals from contaminated water (Hagagg et al., 2020). We speculate that these two bacterial species may be enriched in the uterine microbiota of women with EC/EH as a result of an increased exposure to environmental pollutants. However, there is no published evidence that *Bacillus pseudofirmus* and/or *Stenotrophomonas rhizophila* can cause human infections and/or known disease conditions (Berg and Martinez, 2015; Mukherjee and Roy, 2016; Brooke et al., 2017).

This is, to our knowledge, the first study to demonstrate that these two bacterial species can colonize the human body in general and the uterine cavity in particular. While this was a surprising finding, we believe that the detection of *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* in uterine lavage fluid cannot be considered an artifact for at least two reasons. First, on analyzing bioinformatics data, we implemented a chimera removal step to ensure that any sequencing-related error was removed. Second, we successfully validated the over-representation of the two bacterial species using real-time qPCR in a larger cohort of patients with EC/EH. It is possible that the colonization of the uterine cavity by *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* in this patient group could reflect an interaction between the composition of local microbiota and other variables. An interaction exists when the relationship between an

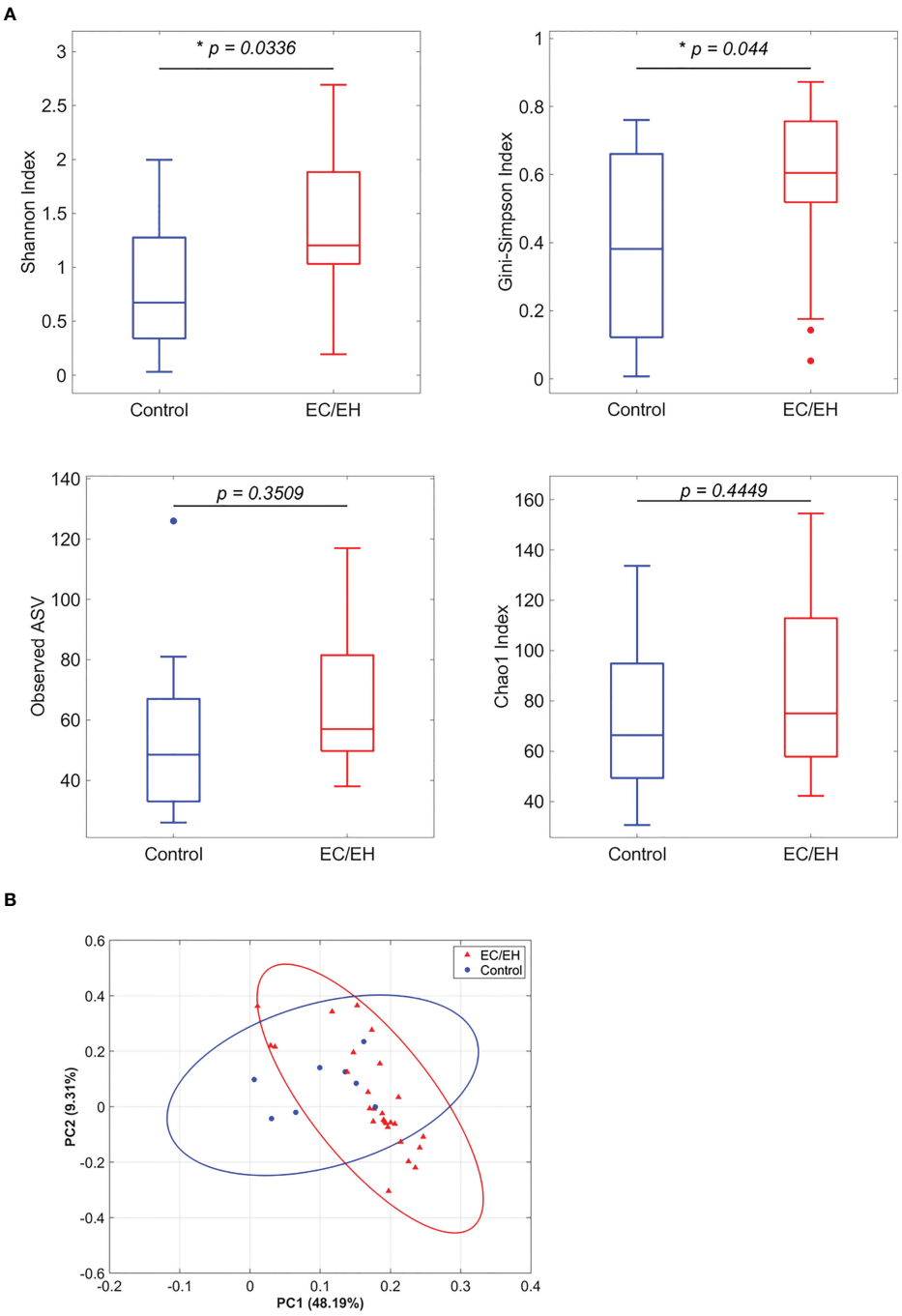

FIGURE 1
Analysis of endometrial lavage microbiota in patients with EC/EH *versus* control women. **(A)** Richness and diversity indices. **(B)** Principal component analysis based on unweighted UniFrac distances. "*" means statistically significant P<0.05.

independent variable x (e.g., contact of the human body with a given bacterial species) and an outcome variable y (e.g., ability of the species to colonize the body after contact and/or causing a disease) varies according to the value of another covariate z (e.g., presence of plastic pollutants). On the one hand, it is plausible that previous studies that failed to detect *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* in the uterine microbiota might have been conducted in women not exposed to plastic pollution. On the other hand, if independently confirmed by future research, *Bacillus pseudofirmus* and *Stenotrophomonas*

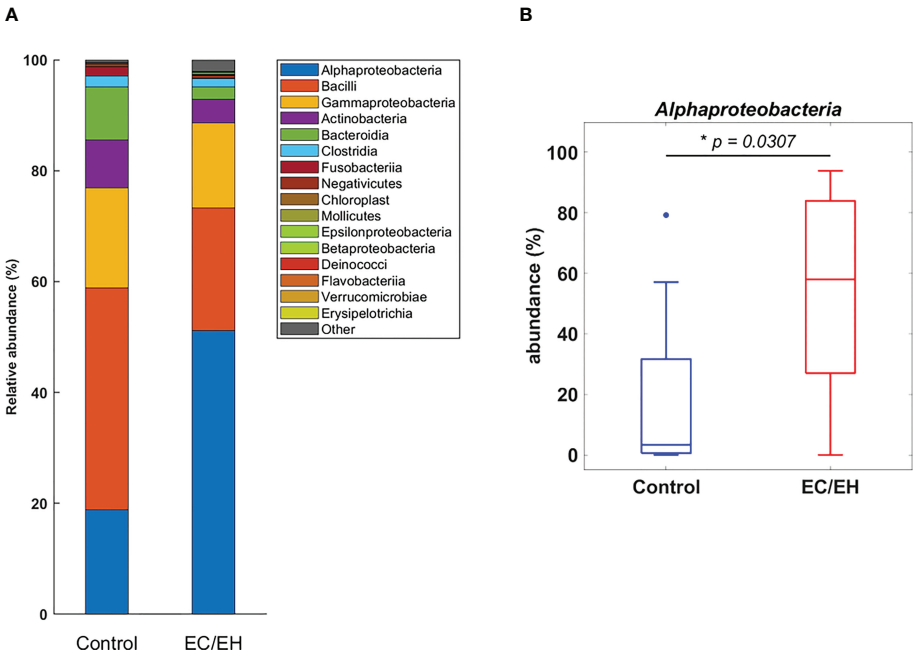

FIGURE 2
(A) Relative abundance of endometrial lavage bacteria in patients with EC/EH *versus* control women: taxonomic identification at the class level. **(B)** Box plots revealed a statistically significant intergroup difference in the relative abundance of the *Alphaproteobacteria* class. "*" means statistically significant P<0.05.

rhizophila may emerge as flagship species for plastic pollution in humans. Importantly, our results highlight the need to include uterine lavage fluid as target samples in studies exploring the impact of plastic contamination on women's health. Both LDPE and certain heavy metals, which are degraded and removed by *Bacillus pseudofirmus* (Dela Torre et al., 2018; Atanasova et al., 2021) and *Stenotrophomonas rhizophila* (Hagagg et al., 2020), respectively, may act as estrogen-mimicking endocrine disruptors to promote endometrial carcinogenesis (Yang et al., 2011; Rzymski et al., 2015). Interestingly, Taiwan is characterized by a markedly high daily intake of estrogen-like pollutants (Lu et al., 2007).

While this pilot study is the first to link the presence of plastic-degrading bacteria in the uterine microbiota with the risk of EC/EH, the higher abundance of *Phyllobacterium* and *Rhodococcus* observed in our study is in accordance with the published literature (Lu et al., 2021). Collectively, these results support the robustness of our analysis. Using computational analysis, we found that the functional profile of endometrial lavage microbiota in EC/EH was associated with fatty acid and amino acid metabolism. Microbiota-derived fatty acids may promote tumorigenesis *via* chronic local inflammation and sustained immune reactions (Mirzaei et al., 2021), whereas amino acids can favor the survival of malignant cells under nutritional, oxidative, and genotoxic stress (Wei et al., 2020). Apart from the detection of *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila*, we also observed that the probiotic bacteria *Lactobacillus* and *Bifidobacterium* were under-represented in the endometrial lavage of patients with EC/EH. This finding is in line with published data (Wieers et al., 2019) and may have contributed to the creation of a carcinogenic milieu through a decreased probiotic-derived production of antitumor molecules (Alizadehmohajer et al., 2020).

Several limitations of our study are worth noting. First, the question as to whether the detection of *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* in uterine lavage fluid is the result of a rapid transit rather than of an effective colonization remains unanswered. A longitudinal investigation with serial sampling of uterine lavage fluid may work to offer a solution to this conundrum. Second, an analysis of microbiota from other body sites in women with EC/EH would have been interesting; unfortunately, as we did not collect these samples for the purpose of the current study, we cannot provide these data. Third, we are currently unable to clarify whether the presence of *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* is the result of environmental contamination or rather it is environmental contamination that affects their behavior. Fourth, it would have been interesting to perform plastic particle

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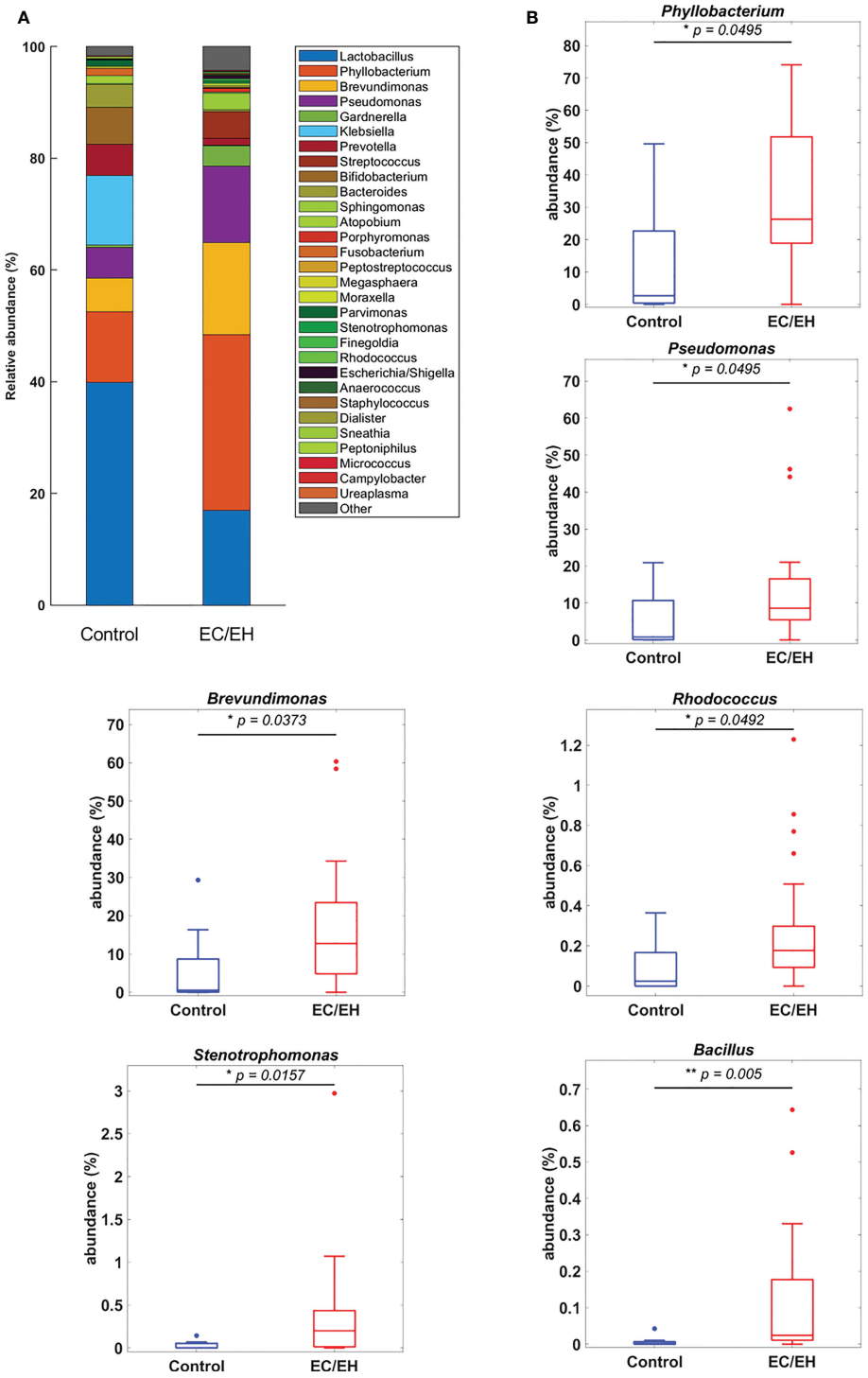

FIGURE 3
(A) Relative abundance of endometrial lavage bacteria in patients with EC/EH *versus* control women: taxonomic identification at the genus level. **(B)** Box plots revealed statistically significant intergroup differences in the relative abundance of the genera *Phyllobacterium*, *Pseudomonas*, *Brevundimonas*, *Rhodococcus*, *Stenotrophomonas*, and *Bacillus*. "*" means statistically significant P<0.05, "**" P<0.01.

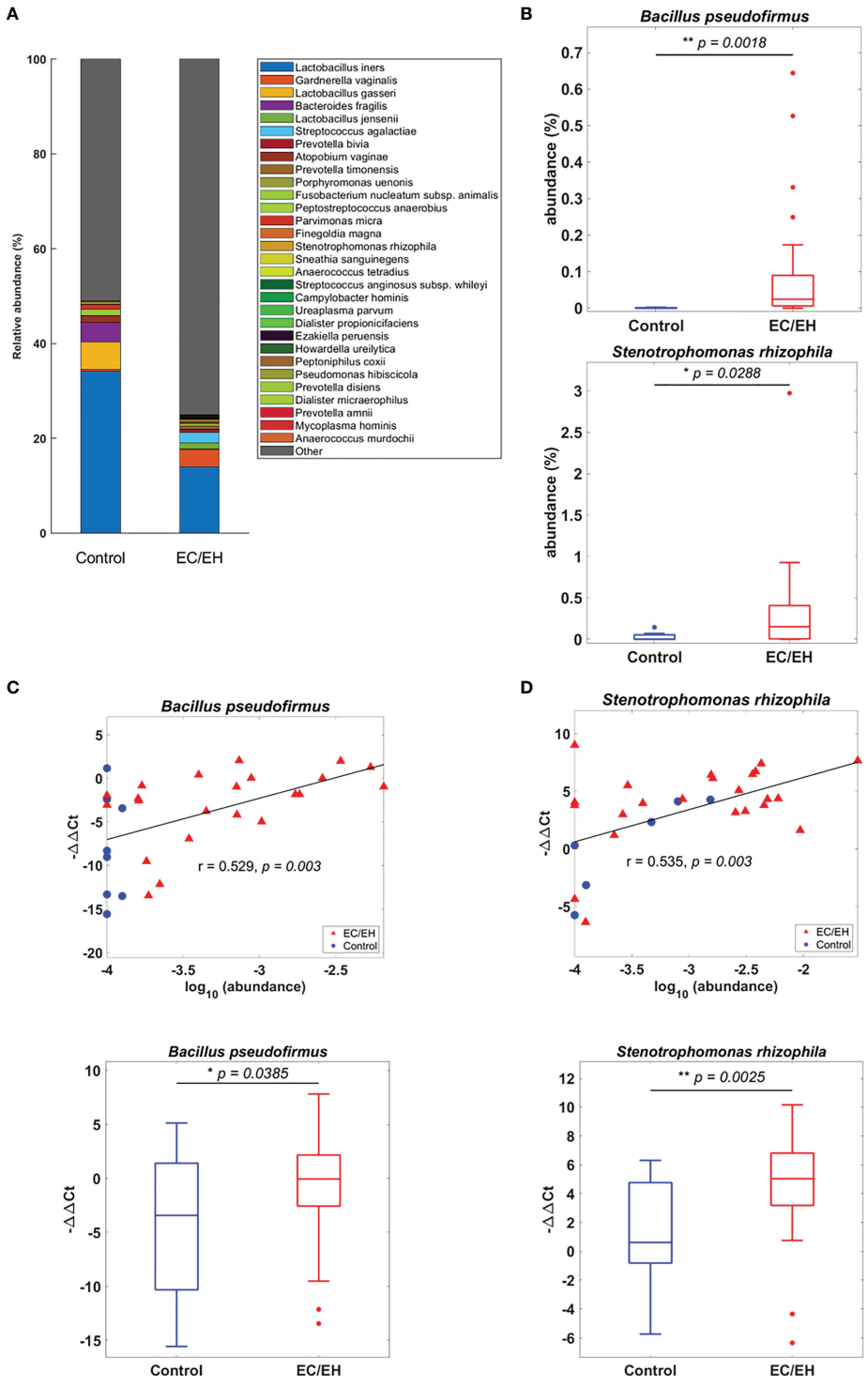

FIGURE 4
(A) Relative abundance of endometrial lavage bacteria in patients with EC/EH *versus* control women: taxonomic identification at the species level. **(B)** Box plots revealed statistically significant intergroup differences in the relative abundance of the species *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila*. **(C)** The higher relative abundance of *Bacillus pseudofirmus* in patients with EC/EH was confirmed by the positive correlation between the results of gene sequencing (r = 0.529, *P* = 0.003; upper panel) and those of real-time qPCR (EC/EH samples, n = 54, control, n = 13, lower panel). **(D)** The higher relative abundance of *Stenotrophomonas rhizophila* in patients with EC/EH was confirmed by the positive correlation between the results of gene sequencing (r = 0.535, *P* = 0.003) and those of real-time qPCR (lower panel). "*" means statistically significant P<0.05, "**" P<0.01.

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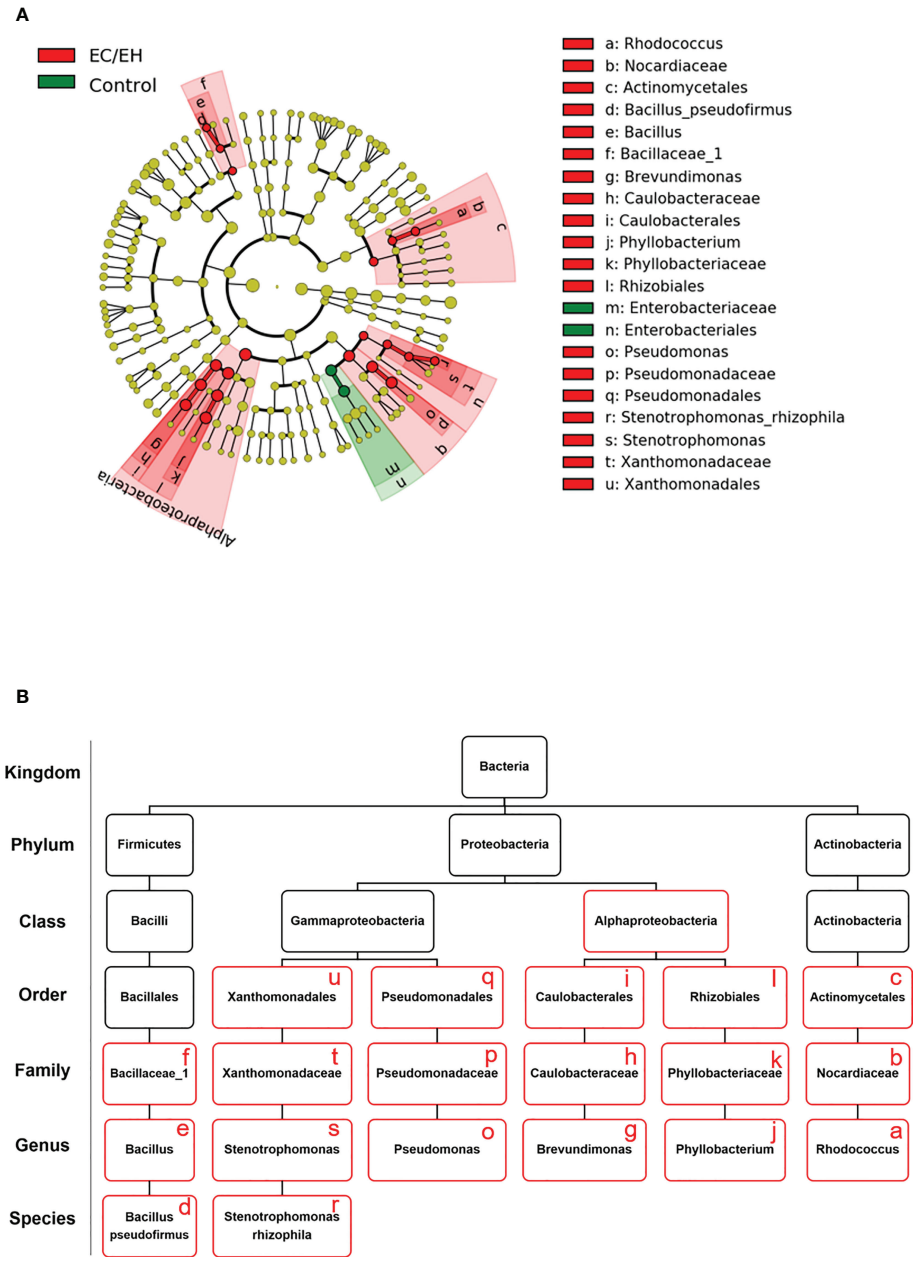

FIGURE 5
Taxonomic profiles of endometrial lavage microbiota in patients with EC/EH *versus* control women: results of linear discriminant analysis of effect size (LEfSe). **(A)** Cladogram plots were generated to visualize significantly enriched bacterial taxa in the two study groups. **(B)** Summary of bacterial taxa showing significant differences between patients with EC/EH *versus* control women (red boxes).

extraction and quantification from the uterine fluid. Unfortunately, our laboratory is currently unable to run the analytical procedure to obtain these data. Finally, we acknowledge the possibility that medical procedures could have acted as a source of plastic contamination in patients with EC/EH.

Conclusions

Our pilot data indicate that the plastic-degrading bacteria *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* are over-represented within the endometrial

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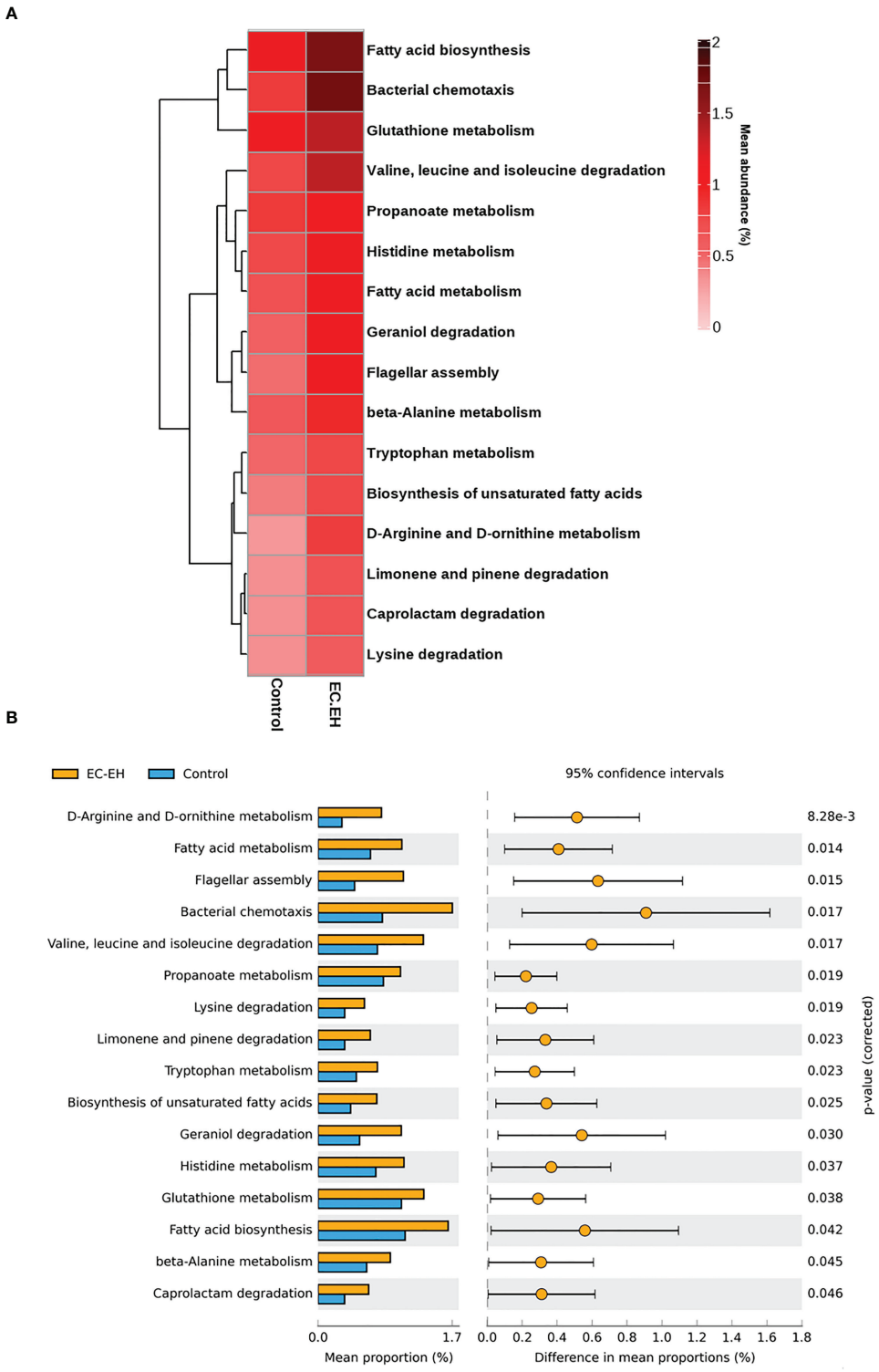

FIGURE 6
PICRUSt prediction of functional pathways in endometrial lavage microbiota of patients with EC/EH *versus* control women. **(A)** Relative abundance of different functional profiles. **(B)** Functional pathways upregulated in patients with with EC/EH.

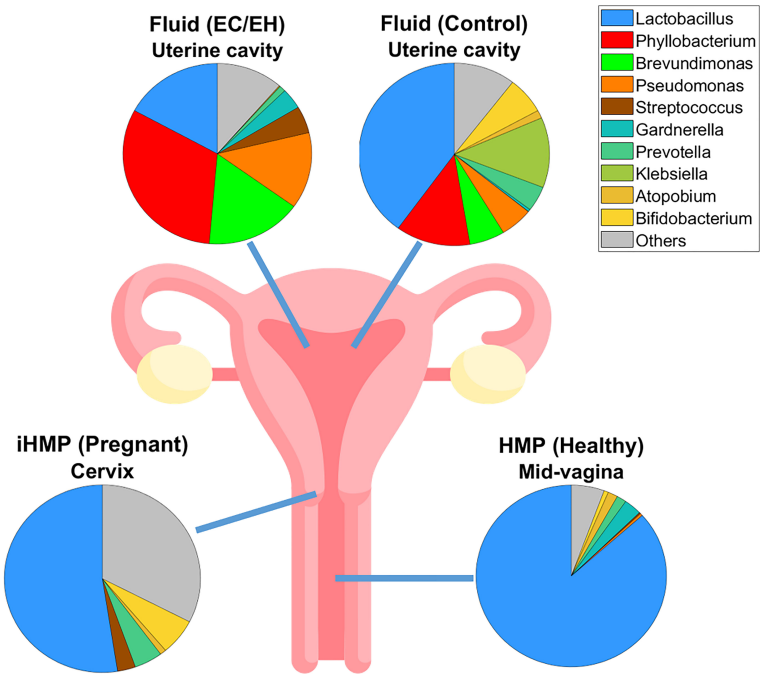

FIGURE 7
Pie plots showing the microbial compositions at the genus level in various levels of the female reproductive tract. A stepwise decrease in the relative abundance of the genus *Lactobacillus* was observed, as follows: 1) mid-vaginal microbiota of healthy non-pregnant women, 2) cervical microbiota of pregnant women, 3) endometrial lavage microbiota of women with benign endometrial lesions, and 4) endometrial lavage microbiota of women with EC/EH. Data on the mid-vaginal microbiota of healthy non-pregnant women and the cervical microbiota of pregnant women were obtained from the Human Microbiome Project (HMP) and the Integrative Human Microbiome Project (iHMP), respectively.

lavage microbiota of women in Taiwan with EC/EH. Despite the intrigue of connecting plastic pollution, uterine microbiota, and different endometrial disease phenotypes, evaluating the hypotheses outlined in our study will require more holistic approaches incorporating serial uterine lavage fluid sampling to identify whether findings are reproducible over time, as well as direct measures of plastic pollution to understand if the observed associations are truly driven by causation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA843535.

Ethics statement

Ethical approval was obtained from the local Institutional Review Board (reference number: 202100083B0) of Chang Gung Memorial Hospital, Taiwan. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AC, K-YW and A-SC: study concept and design; K-YW, C-W, Y-MY, S-SH, Y-SLee, Y-HT, H-JH, Y-SLin, C-HL, A-SC, and C-JW: data collection and interpretation; R-CW: pathological examinations; AC, C-YL, and K-YW: manuscript drafting; AC, A-SC, and C-YL: critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1031967/full#supplementary-material

SUPPLEMENTARY TABLE 1
Characteristics of uterine lavage samples analyzed in the study.

SUPPLEMENTARY TABLE 2
Relative abundance of *Bacillus pseudofirmus* and *Stenotrophomonas rhizophila* according to real-time qPCR results.

SUPPLEMENTARY TABLE 3
Abundance of taxa in patients with EC/EH (n = 27) and control women (n = 8).

SUPPLEMENTARY TABLE 4
Functional prediction of endometrial lavage microbiota in patients with EC/EH (n = 27) and control women (n = 8).

SUPPLEMENTARY TABLE 5
Microbial compositions at the species level in the following groups: 1) mid-vaginal microbiota of healthy non-pregnant women, 2) cervical microbiota of pregnant women, 3) endometrial lavage microbiota of women with benign endometrial lesions, and 4) endometrial lavage microbiota of women with EC/EH.

SUPPLEMENTARY FIGURE 1
Endometrial lavage samples analyzed in this study.

References

Alizadehmohajer, N., Shojaeifar, S., Nedaeinia, R., Esparvarinha, M., Mohammadi, F., Ferns, G. A., et al. (2020). Association between the microbiota and women's cancers - cause or consequences? *BioMed. Pharmacother.* 127, 110203. doi: 10.1016/j.biopha.2020.110203

Atanasova, N., Stoitsova, S., Paunova-Krasteva, T., and Kambourova, M. (2021). Plastic degradation by extremophilic bacteria. *Int. J. Mol. Sci.* 22, 5610. doi: 10.3390/ijms22115610

Baker, J. M., Al-Nakkash, L., and Herbst-Kralovetz, M. M. (2017). Estrogen-gut microbiome axis: Physiological and clinical implications. *Maturitas.* 103, 45–53. doi: 10.1016/j.maturitas.2017.06.025

Berg, G., and Martinez, J. L. (2015). Friends or foes: can we make a distinction between beneficial and harmful strains of the stenotrophomonas maltophilia complex? *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00241

Brooke, J. S., Di Bonaventura, G., Berg, G., and Martinez, J. L. (2017). Editorial: A multidisciplinary look at stenotrophomonas maltophilia: An emerging multi-Drug-Resistant global opportunistic pathogen. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.01511

Chao, A. S., Chao, A., Wang, C. J., Lai, C. H., and Wang, H. S. (2011). Obstetric outcomes of pregnancy after conservative treatment of endometrial cancer: Case series and literature review. *Taiwan. J. Obstet. Gynecol.* 50, 62–66. doi: 10.1016/j.tjog.2009.10.006

Chao, A., Wu, K. Y., Lin, C. Y., Lee, Y. S., Huang, H. J., Tang, Y. H., et al. (2022). Towards less invasive molecular diagnostics for endometrial cancer: massively parallel sequencing of endometrial lavage specimens in women attending for an office hysteroscopy. *J. Mol. Med. (Berl).* 100, 1331–1339. doi: 10.1007/s00109-022-02239-7

Chen, P., Guo, Y., Jia, L., Wan, J., He, T., Fang, C., et al. (2021). Interaction between functionally activate endometrial microbiota and host gene regulation in endometrial cancer. *Front. Cell Dev. Biol.* 9. doi: 10.3389/fcell.2021.727286

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., et al. (2017). The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat. Commun.* 8, 875. doi: 10.1038/s41467-017-00901-0

Chou, W. C., Lee, P. H., Tan, Y. Y., Lin, H. C., Yang, C. W., Chen, K. H., et al. (2017). An integrative transcriptomic analysis reveals bisphenol a exposure-induced dysregulation of microRNA expression in human endometrial cells. *Toxicol. In. Vitro.* 41, 133–142. doi: 10.1016/j.tiv.2017.02.012

Danso, D., Chow, J., and Streit, W. R. (2019). Plastics: Environmental and biotechnological perspectives on microbial degradation. *Appl. Environ. Microbiol.* 85, e01095-19. doi: 10.1128/AEM.01095-19

Dela Torre, D. Y. Z. D., Delos Santos, L. A., Reyes, M. L. C., and Baculi, R. Q. (2018). Biodegradation of low-density polyethylene by bacteria isolated from serpentinization-driven alkaline spring. *Philipp. Sci. Lett.* 11 (Supplement), 1–12.

Dunnick, J. K., Sanders, J. M., Kissling, G. E., Johnson, C. L., Boyle, M. H., and Elmore, S. A. (2015). Environmental chemical exposure may contribute to uterine cancer development: studies with tetrabromobisphenol a. *Toxicol. Pathol.* 43, 464–473. doi: 10.1177/0192623314557335

Felix, A. S., and Brinton, L. A. (2018). Cancer progress and priorities: Uterine cancer. *Cancer Epidemiol. Biomarkers Prev.* 27, 985–994. doi: 10.1158/1055-9965.EPI-18-0264

Gunderson, C. C., Fader, A. N., Carson, K. A., and Bristow, R. E. (2012). Oncologic and reproductive outcomes with progestin therapy in women with endometrial hyperplasia and grade 1 adenocarcinoma: A systematic review. *Gynecol. Oncol.* 125, 477–482. doi: 10.1016/j.ygyno.2012.01.003

Hagagg, K., Hosny, M., Hussien, R., and Rahman, M. A. (2020). Evaluation of environmental pollution and microbial treatment of shallow groundwater in El omayed area, Egypt. *Integr. Environ. Assess. Manage.* 16, 461–471. doi: 10.1002/ieam.4259

Hiroi, H., Tsutsumi, O., Takeuchi, T., Momoeda, M., Ikezuki, Y., Okamura, A., et al. (2004). Differences in serum bisphenol a concentrations in premenopausal normal women and women with endometrial hyperplasia. *Endocr. J.* 51, 595–600. doi: 10.1507/endocrj.51.595

Hubbs, J. L., Saig, R. M., Abaid, L. N., Bae-Jump, V. L., and Gehrig, P. A. (2013). Systemic and local hormone therapy for endometrial hyperplasia and early adenocarcinoma. *Obstet. Gynecol.* 121, 1172–1180. doi: 10.1097/AOG.0b013e31828d6186

Human Microbiome Project C (2012a). A framework for human microbiome research. *Nature.* 486, 215–221. doi: 10.1038/nature11209

Human Microbiome Project C (2012b). Structure, function and diversity of the healthy human microbiome. *Nature.* 486, 207–214. doi: 10.1038/nature11234

Integrative H. M. P. R. N. C (2019). The integrative human microbiome project. *Nature.* 569, 641–648. doi: 10.1038/s41586-019-1238-8

Lear, G., Kingsbury, J. M., Franchini, S., Gambarini, V., Maday, S. D. M., Wallbank, J. A., et al. (2021). Plastics and the microbiome: Impacts and solutions. *Environ. Microbiome.* 16, 2. doi: 10.1186/s40793-020-00371-w

Lin, C. Y., Lin, C. Y., Yeh, Y. M., Yang, L. Y., Lee, Y. S., Chao, A., et al. (2020). Severe preeclampsia is associated with a higher relative abundance of prevotella bivia in the vaginal microbiota. *Sci. Rep.* 10, 18249. doi: 10.1038/s41598-020-75534-3

Lortet-Tieulent, J., Ferlay, J., Bray, F., and Jemal, A. (2018). International patterns and trends in endometrial cancer incidence 1978-2013. *J. Natl. Cancer Inst.* 110, 354–361. doi: 10.1093/jnci/djx2144555056

Lu, Y. Y., Chen, M. L., Sung, F. C., Wang, P. S., and Mao, I. F. (2007). Daily intake of 4-nonylphenol in Taiwanese. *Environ. Int.* 33, 903–910. doi: 10.1016/j.envint.2007.04.008

Lu, W., He, F., Lin, Z., Liu, S., Tang, L., Huang, Y., et al. (2021). Dysbiosis of the endometrial microbiota and its association with inflammatory cytokines in endometrial cancer. *Int. J. Cancer.* 148, 1708–1716. doi: 10.1002/ijc.33428

Lu, L., Luo, T., Zhao, Y., Cai, C., Fu, Z., and Jin, Y. (2019). Interaction between microplastics and microorganism as well as gut microbiota: A consideration on environmental animal and human health. *Sci. Total. Environ.* 667, 94–100. doi: 10.1016/j.scitotenv.2019.02.380

Mallozzi, M., Leone, C., Manurita, F., Bellati, F., and Caserta, D. (2017). Endocrine disrupting chemicals and endometrial cancer: An overview of recent laboratory evidence and epidemiological studies. *Int. J. Environ. Res. Public Health* 14, 334. doi: 10.3390/ijerph14030334

Mirzaei, R., Afaghi, A., Babakhani, S., Sohrabi, M. R., Hosseini-Fard, S. R., Babolhavaeji, K., et al. (2021). Role of microbiota-derived short-chain fatty acids in cancer development and prevention. *BioMed. Pharmacother.* 139, 111619. doi: 10.1016/j.biopha.2021.111619

Mukherjee, P., and Roy, P. (2016). Genomic potential of stenotrophomonas maltophilia in bioremediation with an assessment of its multifaceted role in our environment. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.00967

Nair, N., Camacho-Vanegas, O., Rykunov, D., Dashkoff, M., Camacho, S. C., Schumacher, C. A., et al. (2016). Genomic analysis of uterine lavage fluid detects early endometrial cancers and reveals a prevalent landscape of driver mutations in women without histopathologic evidence of cancer: A prospective cross-sectional study. *PloS Med.* 13, e1002206. doi: 10.1371/journal.pmed.1002206

Rzymski, P., Tomczyk, K., Rzymski, P., Poniedzialek, B., Opala, T., and Wilczak, M. (2015). Impact of heavy metals on the female reproductive system. *Ann. Agric. Environ. Med.* 22, 259–264. doi: 10.5604/12321966.1152077

Salazar, C. A., and Isaacson, K. B. (2018). Office operative hysteroscopy: An update. *J. Minim. Invasive. Gynecol.* 25, 199–208. doi: 10.1016/j.jmig.2017.08.009

Velmurugan, G., Ramprasath, T., Gilles, M., Swaminathan, K., and Ramasamy, S. (2017). Gut microbiota, endocrine-disrupting chemicals, and the diabetes epidemic. *Trends Endocrinol. Metab.* 28, 612–625. doi: 10.1016/j.tem.2017.05.001

Vitale, S. G., Bruni, S., Chiofalo, B., Riemma, G., and Lasmar, R. B. (2020). Updates in office hysteroscopy: a practical decalogue to perform a correct procedure. *Updates. Surg.* 72, 967–976. doi: 10.1007/s13304-020-00713-w

Walther-Antonio, M. R., Chen, J., Multinu, F., Hokenstad, A., Distad, T. J., Cheek, E. H., et al. (2016). Potential contribution of the uterine microbiome in the development of endometrial cancer. *Genome Med.* 8, 122. doi: 10.1186/s13073-016-0368-y

Waring, R. H., Harris, R. M., and Mitchell, S. C. (2018). Plastic contamination of the food chain: A threat to human health? *Maturitas.* 115, 64–68. doi: 10.1016/j.maturitas.2018.06.010

Wei, Y., Fu, J., Wu, J., Jia, X., Zhou, Y., Li, C., et al. (2018). Bioinformatics analysis and characterization of highly efficient polyvinyl alcohol (PVA)-degrading enzymes from the novel PVA degrader stenotrophomonas rhizophila QL-P4. *Appl. Environ. Microbiol.* 84. doi: 10.1128/AEM.01898-17

Wei, Z., Liu, X., Cheng, C., Yu, W., and Yi, P. (2020). Metabolism of amino acids in cancer. *Front. Cell Dev. Biol.* 8, e01898-17. doi: 10.3389/fcell.2020.603837

Wen, H. J., Chang, T. C., Ding, W. H., Tsai, S. F., Hsiung, C. A., and Wang, S. L. (2020). Exposure to endocrine disruptor alkylphenols and the occurrence of endometrial cancer. *Environ. Pollut.* 267, 115475. doi: 10.1016/j.envpol.2020.115475

Wieers, G., Belkhir, L., Enaud, R., Leclercq, S., Philippart de Foy, J. M., Dequenne, I., et al. (2019). How probiotics affect the microbiota. *Front. Cell Infect. Microbiol.* 9. doi: 10.3389/fcimb.2019.00454

Yaguchi, T. (2019). The endocrine disruptor bisphenol a promotes nuclear ERRgamma translocation, facilitating cell proliferation of grade I endometrial cancer cells *via* EGF-dependent and EGF-independent pathways. *Mol. Cell Biochem.* 452, 41–50. doi: 10.1007/s11010-018-3410-0

Yang, B. Y., Gulinazi, Y., Du, Y., Ning, C. C., Cheng, Y. L., Shan, W. W., et al. (2020). Metformin plus megestrol acetate compared with megestrol acetate alone as fertility-sparing treatment in patients with atypical endometrial hyperplasia and well-differentiated endometrial cancer: a randomised controlled trial. *BJOG* 127, 848–857. doi: 10.1111/1471-0528.16108

Yang, C. Z., Yaniger, S. I., Jordan, V. C., Klein, D. J., and Bittner, G. D. (2011). Most plastic products release estrogenic chemicals: A potential health problem that can be solved. *Environ. Health Perspect.* 119, 989–996. doi: 10.1289/ehp.1003220

Zhang, S., Gong, T. T., Liu, F. H., Jiang, Y. T., Sun, H., Ma, X. X., et al. (2019). Global, regional, and national burden of endometrial cancer 1990-2017: Results from the global burden of disease study 2017. *Front. Oncol.* 9. doi: 10.3389/fonc.2019.01440

Zlatnik, M. G. (2016). Endocrine-disrupting chemicals and reproductive health. *J. Midwifery. Womens. Health* 61, 442–455. doi: 10.1111/jmwh.12500

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Xia He,
Nanchang University, China
António Machado,
Universidad San Francisco de Quito, Ecuador
Zhengqiang Hu,
Sichuan University, China

*CORRESPONDENCE
Jun Wang
junwang@im.ac.cn
Bingbing Xiao
doctorxbb@163.com

†These authors have contributed equally to this work

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Vaginal *Lactobacillus iners* abundance is associated with outcome in antibiotic treatment of bacterial vaginosis and capable of inhibiting *Gardnerella*

Rui Zhou[1†], Jingjing Lu[2,3†], Jun Wang[2,3*] and Bingbing Xiao[1*]

[1]Department of Obstetrics and Gynecology, Peking University First Hospital, Beijing, China, [2]Chinese Academy of Science (CAS) Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, [3]University of Chinese Academy of Sciences, Beijing, China

Bacterial vaginosis is characterized as a polymicrobial dysbiosis with the loss of *Lactobacillus* spp. and growth of multiple anerobic bacteria, including *Gardnerella*, *Prevotella* and *Atopobium* ranked as the top three most abundant. A total of nine *Gardnerella* genomospecies have been identified, yet the association between their distribution or any exact *Lactobacillus* species with BV occurrence or prognosis remains controversial. A total of 308 patients and 62 healthy women who sought annual examinations were recruited, with 130 BV patients and 41 healthy women who met our inclusion criteria finally included. Vaginal samples were used for microscopic examination, 16S rRNA sequencing, bacterial culture and isolation. Isolates of *Gardnerella vaginalis*, *Fannyhessae vaginae* (used to be called *Atopobium vaginae*) and *Lactobacillus iners* were used for competition tests. We found that the relative abundances of *Gardnerella*, *Prevotella* and *Atopobium* were elevated in BV patients compared to healthy people ($p<0.0001$), yet no significant differences were found among patients with different clinical outcomes ($p>0.05$). Seven out of nine *Gardnerella* genomospecies were present in both BV patients and healthy women, and the relative abundances of all detected genomospecies were higher in BV patients ($p<0.05$). Cured patients possessed higher GS03 than intermediate and failed patients ($p=0.005$, 0.0337). *L. iners* was significantly higher in cured patients than in the other two groups ($p=0.0021$, $p<0.0001$), and its ability to inhibit the growth of *G. vaginalis* and *F. vaginae* was validated. In summary, seven *Gardnerella* genomospecies were detected in Chinese BV patients, but no association of its distribution and BV occurrence or prognosis was found. The relative abundance of *L. iners* was higher in cured patients, and its antimicrobial activity against *G. vaginalis* and *F. vaginae* was validated through *in vitro* inhibition experiment. *L. iners* could become a predictive indicator of clinical outcomes of BV patients, and its antimicrobial function might be beneficial to BV patients.

KEYWORDS

bacterial vaginosis, *Gardnerella* genomospecies, *Lactobacillus iners*, 16S rRNA sequencing, antimicrobial activity

Introduction

Bacterial vaginosis (BV) is the most common lower genital tract infection, affecting approximately 4-75% of reproductive-aged women internationally (Kenyon et al., 2013; Onderdonk et al., 2016; Abou Chacra et al., 2021). However, the exact etiology of BV still remains unclear. BV is characterized as a dysbiosis of the vaginal microbiome in which the *Lactobacillus* spp. dominant flora is lost (Abou Chacra et al., 2021), accompanied by a significant increase in anaerobic bacteria, including *Gardnerella*, *Atopobium*, *Prevotella*, *Megasphaera*, *Mobiluncus* and so on (Ravel et al., 2011; Muzny et al., 2018; Muzny et al., 2019; Mohankumar et al., 2022). Antibiotics such as metronidazole and clindamycin are recommended for BV treatment, and the short-term cure rate varied from 46.75%-96.20%, but above 70% of women will experience at least one episode of BV recurrence within 12 months (Bostwick et al., 2016; Munoz-Barreno et al., 2021). Recurrent episodes of BV have been demonstrated to be related to a variety of adverse outcomes in gynecology and obstetrics, such as sexually transmitted diseases, cervical cancer, pelvic inflammatory disease, infertility, and premature birth, causing a significant financial burden to the health system and society worldwide (King et al., 2011; Srinivasan et al., 2012; Ravel et al., 2021; Abou Chacra et al., 2021).

In past studies, a variety of anaerobic bacteria have been shown to be closely related to BV. Among these bacteria, *Gardnerella* has attracted special attention, as a series of 16S rRNA sequencing-based techniques have revealed that it could be detected in almost all BV patients, and its presence accounts for the formation of polymicrobial biofilms, which are related to refractory or recurrent BV (Jung et al., 2017; Vestby et al., 2020; Rosca et al., 2022); paradoxically, 40% of healthy women also test positive for such bacteria (Jung et al., 2017). Therefore, whether this species is the contributing pathogen for BV remains debatable (Schwebke et al., 2014; Onderdonk et al., 2016; Morrill et al., 2020). In recent years, researchers have isolated and identified 9 genomospecies of *Gardnerella* through cpn60 gene typing, whole genome sequencing and other methods (Schellenberg et al., 2016; Schellenberg et al., 2017; Hill and Albert, 2019; Vaneechoutte et al., 2019; Qin and Xiao, 2022). Many investigations have focused on identifying the differences in the ability to adhere to vaginal epithelial cells, virulence and drug resistance among genomospecies and the relevance of the distribution of *Gardnerella* genomospecies with the occurrence, symptoms or clinical outcome of BV, but the results lack consistency (Santiago et al., 2011; Onderdonk et al., 2016; Hardy et al., 2017; Hilbert et al., 2017; Deng et al., 2018; Janulaitiene et al., 2018; Ferreira et al., 2021; Khan et al., 2021).

With respect to the normal vaginal microbiome, which is important for the homeostasis of the vaginal environment, studies have also accumulated and concluded that the loss of *Lactobacillus* is an essential part of the progression of BV (Abou Chacra et al., 2021). Based on 16S rRNA sequencing of women across countries and ethnic groups, it is generally accepted that *L. crispatus*, *L. iners*, *L. gasseri* and *L. jensenii* are the four most commonly detected *Lactobacillus* species in the vaginal microbiome (Ravel et al., 2011). Former studies have explored the probiotic effect of different *Lactobacillus* spp. against different pathogens, with *L. crispatus* considered to be the most important species contributing to reproductive health and the combination of *L. gasseri*, *L. jensenii* and *L. acidophilus* might manifest protective effect against dysbiosis of vaginal microbiota (Pacha-Herrera et al., 2022). However, the true role *L. iners* playing in BV progression and prognosis or the restoration of normal vaginal flora remains debatable (Vaneechoutte, 2017; Abou Chacra et al., 2021). Thus, in our study, we profiled the vaginal microbiome in healthy women and BV patients before and after antibiotic treatments and explored the potential contributions of *Gardnerella* and *Lactobacillus* to the treatment outcome of BV at the genomospecies or species level.

Materials and methods

Cohort recruitment

A total of 308 premenopausal (18-50), nonpregnant women who came to Peking University First Hospital with major complaints about vulvovaginal discomfort and/or abnormal vaginal discharge and 62 women who underwent annual physical examinations from August 2020 to August 2021 were recruited for our study and a written form of informed consent was collected, approved and supervised by The Ethics Committee of Peking University First Hospital. All participants underwent blood tests, urine tests and cervicovaginal microscopic or PCR tests to rule out infections of HIV, HPV, HSV-2, syphilis, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, vulvovaginal candidiasis, *Trichomonas vaginalis*, *Ureaplasma urealyticum*, *M. hominis*, urinary tract infections and internal diseases, such as hypertension, diabetes, and hyperlipidemia. Exclusion criteria included pregnancy, diagnosis of any diseases mentioned above, multiple sex partners, history of any intrauterine operations such as hysteroscopy or implantation of intrauterine devices. Patients who were currently in the menstrual period or took oral contraception, any antibiotics whether orally, intravenously or vaginally applied within 30 days or engaged in sexual intercourse within 7 days ahead of sample collection were also excluded from this study. Information of menstrual cycle, last menstruation and reproductive history was also collected by the time of sample collection.

Sample collection

Three vaginal microbiome samples were collected from the same position in the upper 1/3 of the anterior vaginal wall with

vaginal swabs (Becton, Dickinson and Company) during inspection. The first swab was used for DNA extraction and sequencing and immediately stored at -80°C. The second swab was used for Gram staining, microscopic examination and evaluation of biological parameters. The last swab was used for bacterial culture, isolation and purification. The studies involving human participants were reviewed and approved by The Ethics Committee of Peking University First Hospital. All participants signed informed consent in written form for the publication of any potentially identifiable images or data included in this article and agreed to be involved in our follow-up voluntarily.

Diagnostic procedures and treatment

The presence of BV is diagnosed by the Gram stain-based Nugent score according to Nugent et al. (Nugent et al., 1991) (a score of 0-3 is considered to be normal for BV, 4-6 intermediate status, and 7-10 BV) and Amsel criteria according to Amsel et al (Amsel R Fau - Totten et al., 1983) (BV is diagnosed when at least three of the following criteria are fulfilled: Homogenous, thin, grayish-white vaginal discharge that smoothly coats the vaginal wall, vaginal pH>4.5, release of fishy odor when 10% potassium hydroxide is added, and/or over 20% clue cells present on one saline wet mount). Two experienced technicians were involved in the microscopic examination separately and blinded to each other to ensure the authenticity of the diagnosis. Patients who were diagnosed with BV were prescribed topical 5% metronidazole gel for 5 days. All patients were asked to visit their gynecologist again within one week after completion of their treatment. Another two vaginal swabs were collected following the procedures above. The same diagnostic procedures mentioned above were repeated to confirm the patients' clinical outcomes: cured (Nugent 0-3), intermediate (Nugent 4-6) and failed (Nugent 7-10).

Genomic DNA isolation from vaginal samples

The vaginal swab and scraped samples were vortexed and centrifuged for 10 min at 10,000 g to collect the bacterial cells, and the supernatant was discarded. All genomic DNA extractions were performed by using the DNeasy® Power Soil® Pro Kit (Qiagen) following the manufacturer's instructions.

16S rRNA sequencing

A 16S rRNA gene fragment comprising the V3 and V4 hypervariable regions was amplified by using the V3 forward primer 5′-CCTACGGGNBGCASCAG-3′ and the V4 reverse primer 5′-GACTACNVGGGTATCTAATCC-3′. The amplified products were checked and analyzed on a 2% agarose gel. Sequencing was performed using a 250-bp paired-end sequencing protocol on the Illumina NovaSeq6000 platform. Sequence analysis was performed following a previous study (Falony et al., 2016). The sequences were merged using the FLASH program (Magoc and Salzberg, 2011) and subjected to quality filtering using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Chimeras were excluded using the UCHIME command and the 'GOLD' database (Edgar et al., 2011). After random rarefication of microbiome sizes to 6555 reads, the taxonomic assignment of reads was determined by RDP classifier (Wang, 2007) to generate the composition matrices at the level of the phylum to the genus (Wang et al., 2007). The 6,555 rarefied reads were also blasted against the 16S rRNA sequences of established *Gardnerella* genomospecies and *Lactobacillus* species (including *L. crispatus*, *L. iners*, *L. jensenii* and *L. gasseri*) to identify the genomospecies *Gardnerella* and *Lactobacillus*. The 16S rRNA sequences of *Gardnerella* and *Lactobacillus* species in NCBI were collected (Supplementary Table 1) were distinguishable at the species level (Supplementary Figure 1) and were used to build the species level database of *Gardnerella* and *Lactobacillus*. The 6,555 rarefied reads were classified using the BLASTn algorithm against the species level database to identify the species of *Gardnerella* and *Lactobacillus* with identity threshold of >= 99%.

Bacterial isolation and culture conditions

Vaginal swabs were immediately inoculated onto Colombia blood agar, baked sheep blood agar with kanamycin and vancomycin and MRS broth (BD Difcoä) supplemented with IsoVitale XTM Enrichment (BD BBLTM; 2% v/v) and L-Cys (augmented by L-Gln, with a final concentration of 1.1 mM). All broths mentioned above were securely stored at 4°C until used. The broths were placed into an anerobic environment at 37°C using an AS-580 anaerobic chamber (anaerobic system) with an atmosphere of 5% carbon dioxide, 5% hydrogen, and 90% nitrogen (AirgasO) for 24-48 hours. All bacterial colonies from all broths were picked out, purified and identified through 16S sequencing. *G. vaginalis*, *F. vaginae* and *L. iners* were tittered and maintained on Columbia blood agar.

Antimicrobial activity evaluation

Purified *Gardnerella vaginalis* and *Fannyhessae vaginae* (used to be classified into *Atopobium vaginae*) strains were spread onto Colombia blood agar and coated on all boards after activation. Agar containing purchased *L. johnsonii* strains was used as positive control and agar containing purified water was used as negative control. Purchased *L. johnsonii* was safely stored in Microbank tubes (ProLab) at -80°C and fully activated before experiment. Agars containing isolated *L. iners*, purchased

L. johnsonii or water were placed onto broth coated with either *G. vaginalis* (GS01) or *F. vaginae* and cultured under the anaerobic conditions mentioned above. Parallel tests for *L. johnsonii* and *L. iners* were run to ensure the validity of our results. The diameter of the inhibition zone was measured after culturing for 24-48 h.

Statistical analysis

Statistical analysis of bacterial taxonomic identification was performed using R v4.1.1 software. The vegan package was used to analyses the α-diversity and conduct permutational multivariate analysis of variance (PERMANOVA) (Somerfield et al., 2021) followed in case of significant effects by a constrained canonical analysis of principal coordinates (Anderson, 2003). The Wilcoxon test and Kruskal-Wallis test in the ggpubr package were used to measure the difference in richness, α-diversity and abundance.

Results

Cohort description

Of the 370 participants we recruited, 130 BV patients and 41 healthy women were ultimately included in our study. The clinical information of all participants is shown in Table 1. After a standard 5-day metronidazole treatment, patients were divided into three groups according to their clinical outcome: 61 patients were cured (46.9%, group cured), 36 patients turned to intermediate BV (27.7%, group intermediate), and 33 patients still had BV (25.4%, group failed). There was no significant difference in age between healthy women and BV patients (38.03 vs. 37.19, p=0.4764, Kruskal-Wallis test). However, statistically significant differences could be seen in both Nugent score (0.58 vs. 7.88, p<0.0001, Kruskal-Wallis test) and pH (4.21 vs. 5.02, p<0.0001, Kruskal-Wallis test) between the two groups. Moreover, no difference was found in reproductive history or proportion of participants in any menstrual period between healthy participants and BV patients (Table 1). Furthermore, we analyzed the differences in Nugent score and vaginal pH among the three groups before treatment, and no significant differences were found

BV patients have higher *Gardnerella*, *Prevotella* and *Atopobium*

We analyzed all vaginal microbiota through 16S rRNA sequencing (Figure 1). The results reveal that *Lactobacillus.* spp. are the most dominant species in healthy women (78.95%), while 16 (39%) are *L. crispatus* dominant and 14 are *L. iners* dominant (34%). The average relative abundance of *L. crispatus* among those who were *L. crispatus* dominant was 71.77%, while average *L. iners* abundance was 81.27% when *L. iners* was dominant. In contrast, BV-related bacterial species are the most prevalent taxa in BV patients before taking any medications: Gardnerella spp. (35.61%) *Prevotella* (11.66%) and *Atopobium* (10.69%) were among the top three highest relative abundances in BV patients (Table 2). In terms of relative abundance before treatment, all three bacteria were statistically higher than those in healthy women (p<0.0001, Kruskal-Wallis test), while the relative abundance of each bacterium was similar among groups before the application of metronidazole and not statistically significant (p>0.99, Kruskal-Wallis test) (Figure 2).

TABLE 1 Cohort description.

Mean (95CI%)	Healthy participants (N=41)	BV patients (N=130)	P value[b]
Age	38.03 (35.09-40.97)	37.19 (35.64-38.74)	
Nugent Score	0.58 (0.31-0.83)	7.88 (7.65-8.12)	<.0001
pH	4.21 (4.07-4.36)	5.02 (4.96-5.07)	<.0001
Infection of Other STI[a]	None		
Menstrual Cycle			
Follicular phase	48.78% (32.81%-64.75%)	46.15% (37.47%-54.84%)	
Period of ovulation	17.07% (5.05%-29.10%)	6.92% (2.50%-11.35%)	
Luteal phase	34.15% (18.99%-49.30%)	46.92% (38.23-55.62%)	
Reproductive history			
G	1.39 (1.07-1.71)	1.45 (1.24-1.67)	
P	0.71 (0.55-0.87)	0.80 (0.69-0.91)	

BV, bacterial vaginosis; STI, sexually transmitter infections.
[a]Other STI include HIV, HPV, HSV-2, syphilis, Chlamydia trachomatis, Neisseria gonorrhoeae, vulvovaginal candidiasis, Trichomonas vaginalis, Ureaplasma urealyticum, M. hominis, and urinary tract infections. [b]Only statistically significant P value is manifested in the table. Kruskal-Wallis test was used for inter-group comparison.

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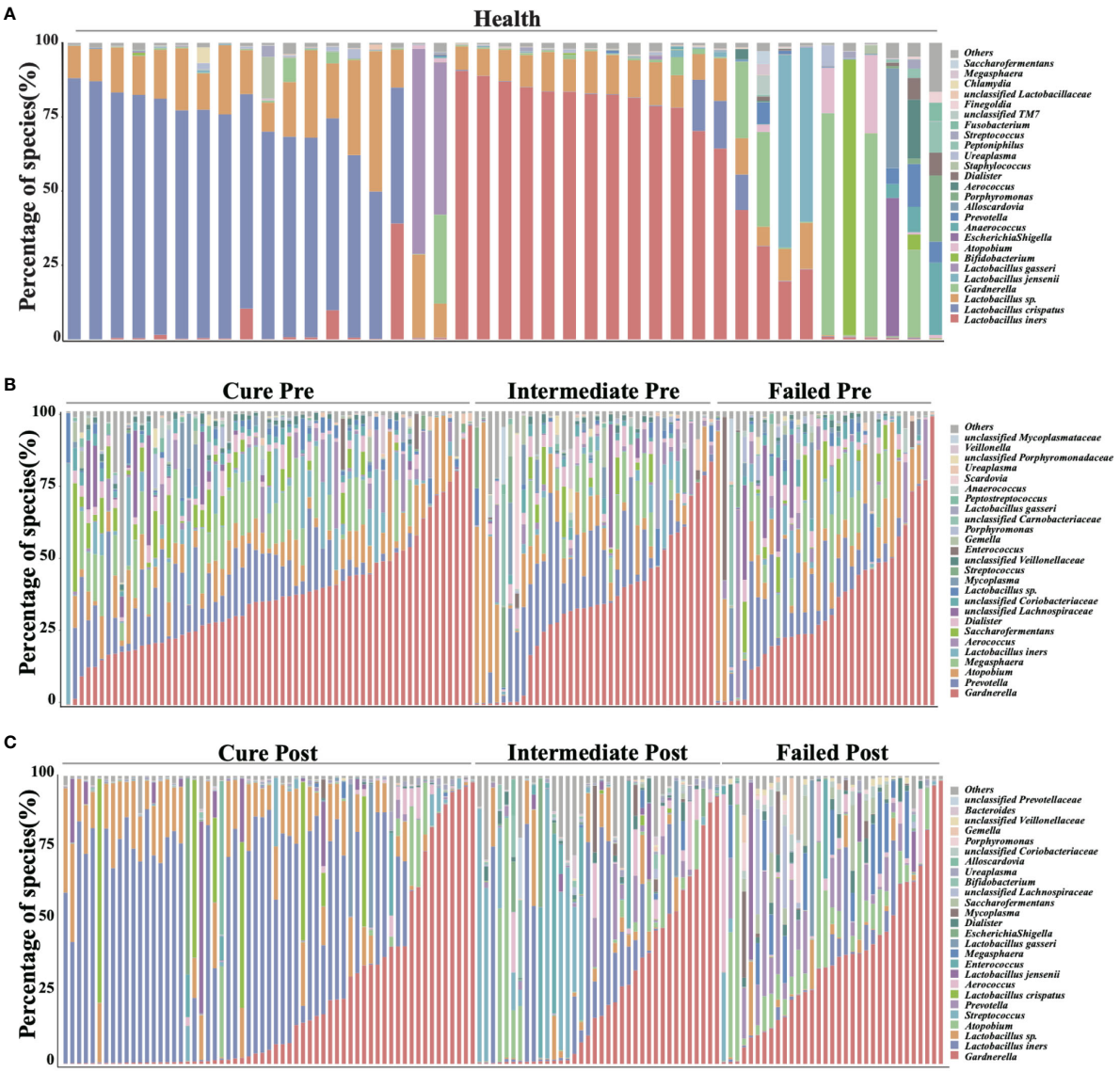

FIGURE 1
Vaginal microbiome composition of studied cohort. This figure manifests the top 26 most abundant bacteria in participants' vaginal microbiome, organisms ranked 27 and below are all categorized into label "others". **(A)** shows the vaginal microbiome of healthy participants, with **(B)** showing BV patients pretreatment and **(C)** showing posttreatment.

We then analyzed the vaginal microbiome in BV patients after metronidazole treatment and found significant differences in microbiome composition among patients in different clinical outcome groups. In cured patients (group cured), the relative abundance of all three BV-associated bacteria significantly decreased ($p<0.0001$, Wilcoxon test), yet the relative abundance of Gardnerella spp. was still higher than that in the healthy cohort ($p<0.0001$, Wilcoxon test). The relative abundance of both *Atopobium* and *Prevotella* decreased posttreatment in the intermediate group (group intermediate, $p=0.0103$, $p<0.0001$, Wilcoxon test), but the abundance of Gardnerella spp. did not change significantly ($p=0.0946$, Wilcoxon test). In contrast to the two groups with improvement, no significant decrease in any bacteria was detected in patients without improvement (failed group). Intergroup comparison shows that patients in the cured group had a lower relative abundance of Gardnerella spp., *Atopobium* and *Prevotella* than the failed group ($p=0.0009$, $p<0.0001$, $p<0.0001$, Kruskal-Wallis test) and a lower relative abundance of *Atopobium* and *Prevotella* than the intermediate group

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TABLE 2 Relative abundance of top 10 most abundant bacteria at genus level.

Genus	All participants (N=171)			Group cured (N=61)[a]			Group intermediate (N=36)[b]			Group failed (N=33)[c]		
	Healthy	BV patients	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]
Relative abundance (%) of each genus, mean (95%CI)												
Lactobacillus	78.95 (68.00-89.89)	7.66 (5.16-10.15)	<.0001	10.06 (5.96-14.16)	67.33 (57.78-77.09)	.0048	4.96 (1.53-8.38)	11.46 (4.29-18.64)	.231	6.23 (0.92-11.54)	23.23 (12.06-34.39)	.0761
Gardnerella	7.12 (1.70-12.54)	35.61 (31.73-39.50)	<.0001	37.21 (31.93-42.50)	22.32 (14.33-30.30)	<.0001	34.08 (26.07-42.09)	25.74 (15.97-35.51)	.0946	34.38 (25.73-43.03)	36.01 (26.72-45.30)	.980
Prevotella	0.98 (0.10-1.85)	11.66 (9.64-13.68)	<.0001	10.73 (7.97-13.48)	0.33 (0.10-0.56)	<.0001	14.07 (9.61-18.52)	2.69 (0.78-4.60)	<.0001	10.74 (6.69-14.78)	9.09 (4.99-13.19)	.1482
Atopobium	1.25 (-0.23-2.73)	10.69 (8.08-13.31)	<.0001	8.27 (6.24-10.29)	1.95 (0.29-3.61)	<.0001	12.35 (5.85-18.85)	7.02 (1.33-12.71)	.0103	13.29 (6.54-20.04)	12.07 (5.70-18.45)	.0657
Megasphaera	0.12 (-0.06-0.30)	7.56 (6.04-9.08)	<.0001	9.08 (6.74-11.42)	0.10 (0.045-0.16)	<.0001	5.74 (2.95-8.53)	0.61 (-0.05-1.27)	.0005	6.78 (3.81-9.76)	6.85 (3.56-10.15)	.7341
Aerococcus	0.60 (-0.40-1.59)	3.77 (2.35-5.18)	<.0001	3.63 (2.02-5.24)	0.93 (0.36-1.50)	<.0001	3.14 (0.81-5.48)	3.38 (1.16-5.60)	.6507	4.70 (0.52-8.89)	4.76 (0.60-8.91)	.7609
Saccharofermentans	0.12 (-0.096-0.33)	3.26 (2.27-4.25)	<.0001	4.09 (2.40-5.79)	0.04 (0.017-0.060)	<.0001	2.06 (0.58-3.54)	0.15 (0.02-0.29)	.0013	3.06 (1.23-4.90)	2.95 (0.91-4.99)	.8656
Dialister	0.30 (-1.96-2.55)	2.95 (2.52-3.39)	<.0001	3.33 (-6.40-13.05)	0.09 (-0.50-0.67)	<.0001	2.90 (-7.85-13.66)	0.81 (-7.53-9.14)	<.0001	2.93 (1.23-4.62)	2.82 (1.20-4.44)	.5207
Streptococcus	0.31 (0.045-0.57)	0.95 (0.23-1.68)	<.0001	0.20 (0.019-0.39)	1.13 (0.15-2.11)	.1953	2.28 (0.098-4.47)	13.60 (4.64-22.56)	.0123	0.86 (-0.71-2.43)	1.14 (-0.75-3.04)	.5391
Enterococcus	0.02 (0.004-0.05)	0.82 (-0.10-1.74)	.290	0.12 (0.030-0.320	0.00 (0.00-0.002)	.0143	0.08 (0.0015-0.16)	0.00 (0.00-0.01)	.009	1.99 (-1.46-5.44)	1.15 (-0.97-3.28)	.7718

BV, bacterial vaginosis.
[a] "Group Cured" was defined as patients whose Nugent score were lowered to 0-3 after metronidazole treatment. [b] "Group Intermediate" was defined as patients whose Nugent score were changed to 4-6 after metronidazole treatment. [c] "Group Failed" was defined as patients whose Nugent score remained at 7-10 after metronidazole treatment. [d] Wilcoxon test was used for comparison between these two groups.

(p=0.0002, p=0.0038, Kruskal-Wallis test). Meanwhile, the intermediate group contained a lower relative abundance of *Atopobium* and *Prevotella* than the failed group (p=0.0022, p=0.0254, Kruskal-Wallis test), but the relative abundance of Gardnerella spp. showed no significant differences between the cured and intermediate groups or between the intermediate and failed groups (Figure 2).

Furthermore, we analyzed the α-diversity (Shannon index and Chao1 index) in participants' vaginal microbiota (Supplementary Figure 3). We found that, α-diversity in all BV patients was significantly higher compared to healthy women (p<0.05), yet no statistical differences were noticed among groups with different clinical outcomes pretreatment. After metronidazole treatment, α-diversity was reduced in group cured and significantly higher than group intermediate and group failed, but was still significantly higher compared to healthy women. No statistical changes of α-diversity were noticed in either group intermediate or group failed after treatment. Moreover, no statistical differences were shown between group intermediate and group failed post-treatment.

Cured patients possessed higher GS03 pretreatment

Since former studies have recognized nine different *Gardnerella* genomospecies *via* whole genome sequencing, only seven genomospecies have been detected in our specimen, namely, GS01, GS02, GS03, GS05, GS07, GS08 and GS09, with decreasing abundance. Each detected *Gardnerella* genomospecies was increased in BV patients compared to healthy women pretreatment (p<0.01, Kruskal-Wallis test) (Table 3). When comparing groups of patients with different treatment outcomes, we found that only the abundance of GS03 in the cured group was significantly higher than that in the intermediate group and the group that failed before treatment (p=0.005, 0.0337, Kruskal-Wallis test), while the abundances of other genomospecies showed no significant differences among groups (Figure 3).

With respect to treatment outcome, in the cured group, the relative abundance of every *Gardnerella* genomospecies was decreased posttreatment (p<0.05, Wilcoxon test), but only the relative abundance of GS07, GS08 and GS09 was restored to levels similar to those of healthy individuals (Figure 3). In the intermediate group or the failed group, no significant changes were found in any genomospecies before and after treatment (p>0.05, Wilcoxon test). Further analysis showed that the relative abundance of GS05, GS07 and GS08 was lower in the cured group than in the intermediate group, and all genomospecies were significantly lower than in the failed group. Between the intermediate group and the failed group, only GS03 showed significant differences (p=0.0265, Kruskal-Wallis test) (Figure 3).

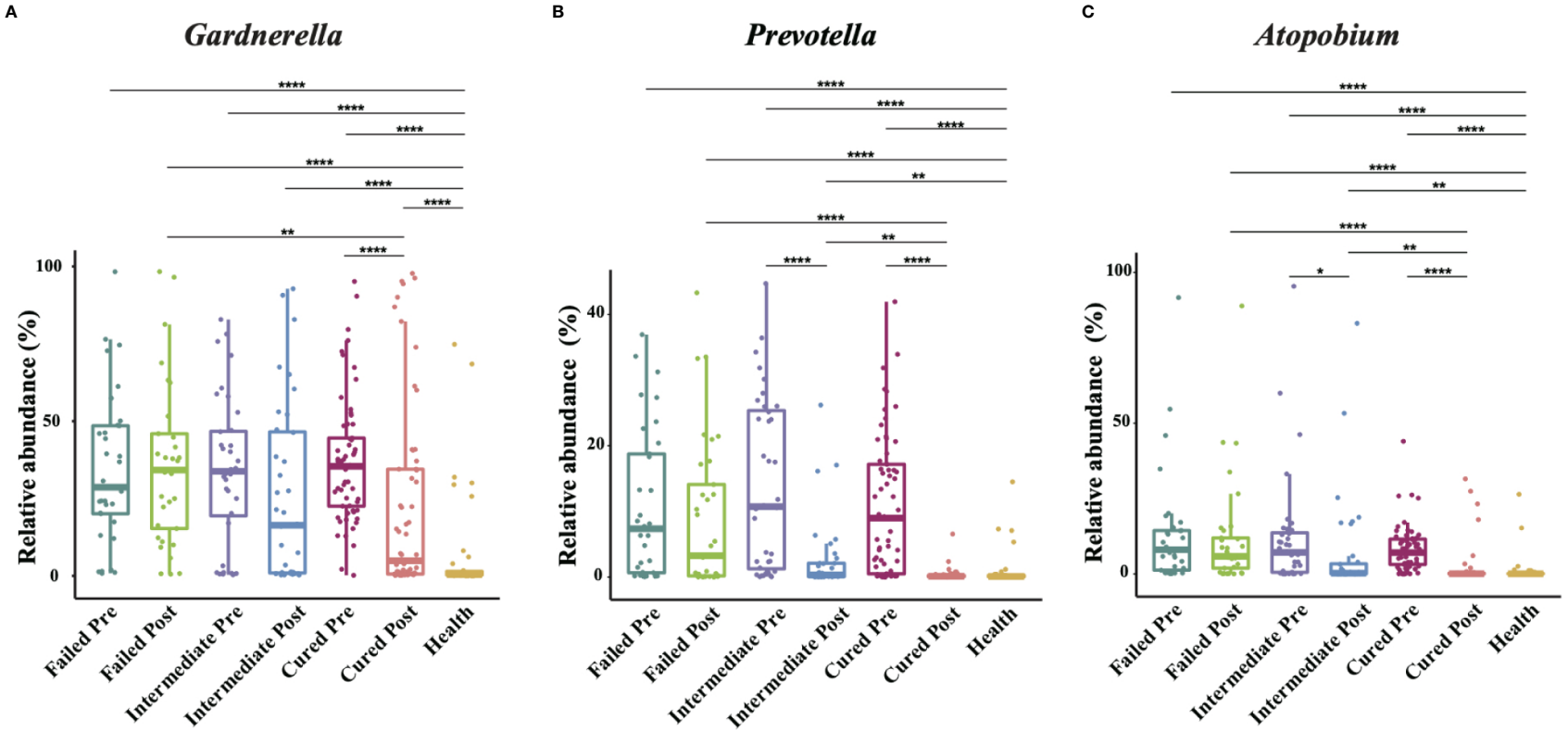

FIGURE 2
Comparison of *Gardnerella, Prevotella* and *Atopobium* abundance in different groups. Intergroup comparison of *Gardnerella* **(A)**, *Prevotella* **(B)** and *Atopobium* **(C)** relative abundance. Only statistically significant *P* value that has clinical meaning is marked in the graph. Significance is exhibited as: *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; Wilcoxon test for pairwise comparison between pre- and posttreatment and Kruskal-Wallis test for comparisons among different groups.

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TABLE 3 Relative abundance of each *Gardnerella* genomospecies in each group.

Genomospecies	All participants (N=171)			Group cured (N=61)[a]			Group intermediate (N=36)[b]			Group failed (N=33)[c]		
	Healthy	BV patients	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]
Relative abundance (%) of each genus, mean (95%CI)												
GS01	5.20 (0.37-10.02)	14.7 (11.14-18.26)	<.0001	15.22 (9.89-20.54)	11.03 (5.47-16.59)	.0002	12.17 (5.63-18.71)	12.94 (6.09-19.79)	.3272	16.52 (8.84-24.19)	17.95 (9.41-26.48)	.8878
GS02	0.65 (0.14-1.16)	9.16 (6.98-11.33)	<.0001	7.94 (5.54-10.34)	3.73 (1.13-6.32)	<.0001	11.66 (6.00-17.31)	7.56 (3.18-11.95)	.2112	8.64 (4.33-12.95)	9.04 (4.56-13.53)	.9234
GS03	0.54 (-0.16-1.23)	2.83 (1.89-3.76)	<.0001	3.84 (2.30-5.37)	3.45 (0.77-6.13)	<.0001	1.62 (0.37-2.86)	0.68 (0.10-1.26)	.5614	2.30 (0.30-4.30)	2.16 (0.42-3.89)	.8374
GS05	0.02 (0.00-0.03)	2.17 (1.56-2.77)	<.0001	2.35 (1.37-3.33)	0.52 (0.03-1.01)	.0012	1.95 (0.93-2.96)	1.48 (0.35-2.62)	.5835	2.07 (0.84-3.30)	1.93 (0.84-3.01)	.657
GS07	0.05 (-0.05-0.15)	0.52 (0.16-0.87)	.0053	0.42 (0.10-0.74)	0.00 (0.00-0.00)	.0053	0.85 (-0.24-1.93)	0.03 (0.00-0.05)	.6835	0.33 (-0.20-0.87)	0.62 (-0.05-1.75)	.7967
GS08	0.00 (0.00-0.00)	0.01 (0.00-0.02)	.0189	0.02 (0.00-0.03)	0.00 (0.00-0.00)	<.0001	0.02 (-0.02-0.04)	0.02 (-0.02-0.04)	.2555	0.00 (0.00-0.00)	0.02 (0.00-0.03)	.6641
GS09	0.01 (-0.01-0.02)	0.28 (0.22-0.35)	<.0001	0.32 (0.21-0.43)	0.09 (0.02-0.16)	<.0001	0.27 (0.16-0.37)	0.18 (0.05-0.31)	.0954	0.24 (0.11-0.37)	0.21 (0.10-0.31)	.9843

BV, bacterial vaginosis; GS, genomospecies.
[a] "Group Cured" was defined as patients whose Nugent score were lowered to 0-3 after metronidazole treatment. [b] "Group Intermediate" was defined as patients whose Nugent score were changed to 4-6 after metronidazole treatment. [c] "Group Failed" was defined as patients whose Nugent score remained at 7-10 after metronidazole treatment. [d] Wilcoxon test was used for comparison between these two groups.

Higher *L. iners* is associated with a positive outcome of BV treatment

The four most commonly observed *Lactobacillus* species in reproductive-aged women are *L. crispatus*, *L. iners*, *L. gasseri* and *L. jensenii;* we specifically allocated the sequences to the four species with a stringent similarity threshold (99%). In the results, we found *L. iners* to be the highest in terms of abundance in healthy individuals, with *L. crispatus*, *L. jensenii* in decreasing order and *L. gasseri* having the lowest proportion. The relative abundance of *Lactobacillus* spp. in BV patients was overall significantly lower than that in healthy group pre-treatment, but in terms of species, only *L. crispatus* and *L. iners* were significantly different among BV patients and healthy women ($p<0.0001$, $p=0.0407$, Kruskal-Wallis test) (Table 4). We also discovered that even though the relative abundance of *Lactobacillus* spp. in total among the three groups of BV patients was similar before treatment, but the proportion of *L. iners* was higher in the cured group than in the intermediate and failed pretreatment groups ($p=0.0021$, $p<0.0001$, Kruskal-Wallis test), while it was not significantly different between the intermediate and failed groups ($p>0.9999$, Kruskal-Wallis test) (Figure 4).

In addition, we found that *Lactobacillus* spp. abundance in total was restored only in the group cured after being treated with metronidazole ($p=0.0048$, Wilcoxon test), while other two groups showed no signs of *Lactobacillus* spp. restoration. But at the species level, only *L. iners* showed a significant difference ($p=0.0007$, Wilcoxon test); thus, it was the most affected species. After metronidazole treatment, only the relative abundance of *L. iners* was significantly different among treatment outcome groups, as the cured group possessed a higher *L. iners* relative abundance than the intermediate and failed groups ($p=0.02$, $p=0.0274$, Kruskal-Wallis test). No difference was found between the intermediate group and the failed group with regard to any other *Lactobacillus* species abundance ($p>0.05$, Kruskal-Wallis test) (Figure 4).

Lactobacillus iners inhibits GS01 and Fennyhessae vaginae *in vitro*

As our results indicate that higher *L. iners* is associated with a positive outcome of BV treatment, we examined whether *L. iners* possessed antimicrobial ability against BV related bacteria. We co-cultured clinically isolated *L. iners* with GS01 or *F. vaginae* and used *L. johnsonii*, which has been reported to be capable of inhibiting the growth of a series of pathogens, as a positive control and agar containing purified water as negative control. We found that after culturing for 24-48 h, inhibition zones were manifested in all parallel tests cocultured with GS01 or *F. vaginae*, indicating the inhibitory effect of *L. iners* against

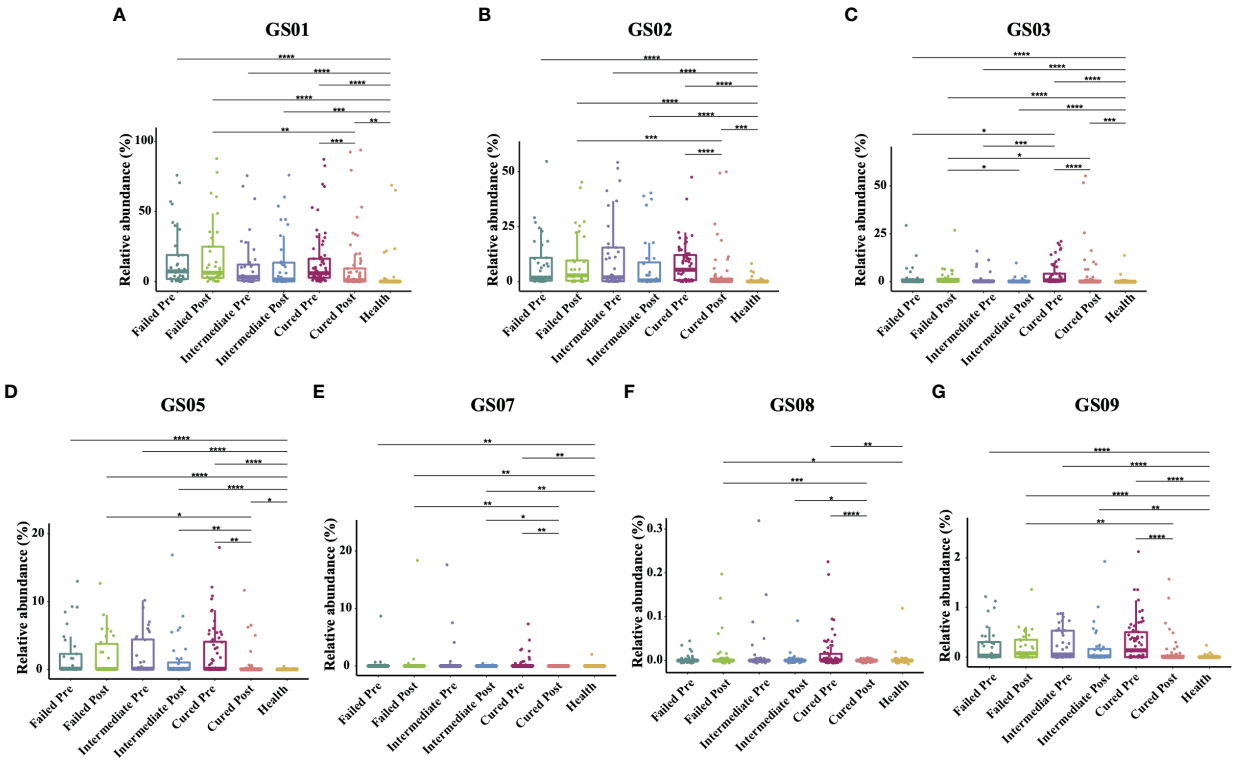

FIGURE 3
Comparison of *Gardnerella* genomospecies abundance in different groups. Intergroup comparison of the relative abundance of each *Gardnerella* genomospecies: GS01 **(A)**, GS02 **(B)**, GS03 **(C)**, GS05 **(D)**, GS07 **(E)**, GS08 **(F)** and GS09 **(G)**. Only statistically significant *P* value that has clinical meaning is marked in the graph. Significance is exhibited as: *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; Wilcoxon test for pairwise comparison between pre- and posttreatment and Kruskal-Wallis test for comparisons among different groups.

the growth of GS01 and *F. vaginae* in the *in vitro* coculture system, yet system of negative control showed no sign of antimicrobial abilities. (Supplementary Figure 4).

Discussions

Our study shows that *L. iners* (30.33%), *L. crispatus* (29.21%) and *Gardnerella* (7.12%) ranked the top three most prevalent bacteria in healthy Chinese women, with *Gardnerella* (37.12%), *Prevotella* (10.73%) and *Atopobium* (8.72%) ranked as the top three in BV patients, but no such correlation between the relative abundance of *Gardnerella*, *Atopobium* or *Prevotella* and clinical outcomes was found. Interestingly, even though the amount of healthy individuals dominant by *L. crispatus* was slightly higher than those dominant by *L. iners* (16 vs 14), we noticed that the average relative abundance of *L. crispatus* was even mildly lower than *L. iners* (29.21% vs 30.33%). This was due to the average abundance of dominant *Lactobacillus* species was different. It seems that when *L. iners* is the dominant species in vaginal flora, it occupies more ecological niche than *L. crispatus*. Our study also showed that BV patients had higher richness and diversity compared to healthy women pretreatment which is in consistence with former studies. The reduction of diversity in group cured after treatment infers the success of treatment, yet the richness is still significantly higher than healthy women indicates that further therapeutic procedures might be required to fully restore the normal vaginal microbiota.

Former studies have demonstrated that different genomospecies of Gardnerella spp. manifest diverse characteristics such as virulent factors, adherent abilities, antibiotics resistance, etc. Although lacking explicit conclusions, studies based on cohorts from other regions and ethnicities, utilizing the concept of "*Gardnerella* clades" based on cpn60 sequencing reported that different structures of the *Gardnerella* clades are related to BV, as certain genomospecies being more abundant or positive in BV patients, while others are not (Numanović et al., 2008; Vodstrcil et al., 2017). In our study, we brought former studies to a further level as we analyzed *Gardnerella* genomospecies which were classified by whole-genome sequencing. We detected seven out of nine genomospecies in Chinese women, with the absence of GS04 and GS06 and all detected genomospecies were presented in both BV patients and healthy people. Furthermore, GS01, GS02,

TABLE 4 Relative abundance of four *Lactobacillus* species in each group.

Species	All participants (N=171)			Group cured (N=61)[a]			Group intermediate (N=36)[b]			Group failed (N=33)[c]		
	Healthy	BV patients	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]	Pretreatment	Posttreatment	*P* value[d]
Relative abundance (%) of each genus, mean (95%CI)												
L. crispatus	29.21 (18.01-40.41)	0.08 (0.06-0.11)	<.0001	0.069 (0.051-0.087)	5.67 (1.25-10.09)	.4657	0.11 (0.041-0.18)	0.22 (0.0012-0.44)	.4011	0.08 (0.024-0.14)	0.081 (0.047-0.11)	.2359
L. iners	30.33 (18.69-41.41)	4.83 (3.12-6.54)	.0288	7.31 (3.89-10.64)	43.71 (34.88-52.54)	.0007	2.51 (0.74-4.27)	15.65 (7.22-24.09)	.0989	2.86 (1.01-4.72)	3.49 (0.68-6.30)	.6350
L. gasseri	2.99 (-1.20-7.19)	0.51 (-0.14-1.15)	.0288	0.44 (-0.16-1.04)	1.64 (-0.60-3.87)	.596	1.08 (-1.08-3.23)	0.95 (-0.91-2.81)	.9237	0.01 (0.0025-0.025)	1.67 (-1.67-5.01)	.3355
L. jensenii	3.17 (-1.09-7.43)	0.31 (-0.20-0.82)	.2024	0.03 (0.014-0.047)	2.79 (0.25-5.32)	.8006	0.064 (0.018-0.11)	1.23 (-0.30-2.77)	.7226	1.08 (-0.96-3.12)	2.82 (-1.15-6.79)	.9946

BV, bacterial vaginosis.
[a] "Group Cured" was defined as patients whose Nugent score were lowered to 0-3 after metronidazole treatment. [b] "Group Intermediate" was defined as patients whose Nugent score were changed to 4-6 after metronidazole treatment. [c] "Group Failed" was defined as patients whose Nugent score remained at 7-10 after metronidazole treatment. [d] Wilcoxon test was used for comparison between these two groups.

and GS03 ranked the top three most prevalent and GS08 the least in both BV patients and healthy people. No genomospecies are thought to be specifically related to BV, as the relative abundance of all genomospecies is significantly higher in BV patients than in healthy women.

A previous study reported an association between a high abundance of certain *Gardnerella* genomospecies or a combination of several genomospecies with BV clinical outcomes (Harwich et al., 2010; Hilbert et al., 2017; Faught and Reyes, 2019; Hill and Albert, 2019; Shipitsyna et al., 2019), and coinfection of GS03 and GS04 was thought to be related to negative clinical outcomes based on a cohort of recurrent BV patients (Turner et al., 2021; Qin and Xiao, 2022). In contrary to former studies, our study found that the relative abundance of GS03 was even higher in cured patients. However, whether GS03 relative abundance is associated with better clinical outcomes might remain controversial, as in our study, GS01 is the most prevalent *Gardnerella* genomospecies and former studies have shown that GS01 is less resistant to metronidazole compared to GS03 (35% vs 100%). GS03 only made up 3.84% of the whole bacterial taxa which was approximately 1/5 of the most abundant genomospecies GS01(14.7%) and it's difficult to determine whether this small amount of GS03 is able to shift the clinical outcomes of BV patients. Therefore, we propose that GS01 instead of GS03 might be the most important genomospecies affecting BV clinical outcomes.

At the same time, our study noticed that patients with more *L. iners* before treatment might have a better clinical outcome. This is a notable finding, as it indicates that *L. iners* may be an innovative indicator for BV clinical outcomes, in contrast to previous findings that it might be an opportunistic pathogen (Lee et al., 2020). Different from other *Lactobacillus* species mentioned in this article, *L. iners* shows unique metabolic and genomic characteristics, and its protective function is questionable compared to other *Lactobacilli* (France et al., 2016; France et al., 2020; Bloom et al., 2022; France M. T. et al., 2022). Its production of hydrogen peroxide and D-lactic acid is lower, and the inerolysin it secretes is thought to be a cholesterol-dependent cytotoxin that is homogenous to vaginolysin and expressed by several BV-associated bacteria (Zhou et al., 2010; Pleckaityte, 2019; Ragaliauskas et al., 2019; Zheng et al., 2021). It has been acknowledged that metronidazole instantly reduces the load of vaginal microbiota, and *L. iners* becomes the dominant species (Lehtoranta et al., 2020; Verwijs et al., 2020; Armstrong et al., 2022), but this kind of structure is unstable and has the potential to lead to BV recurrence (France M. et al., 2022). Although lacking consistent conclusions, *L. iners* is considered to be a "foe" instead of a "friend" (Hill and Albert, 2020; Zheng et al., 2021; Novak et al., 2022). However, our study proposed a novel point of view, as we found that the cured patients' microbiome had more *L. iners*. Our *in vitro* experiments also validated the inhibitory effect of *L. iners* against *G. vaginalis* and *F. vaginae*. We assume that when *L.*

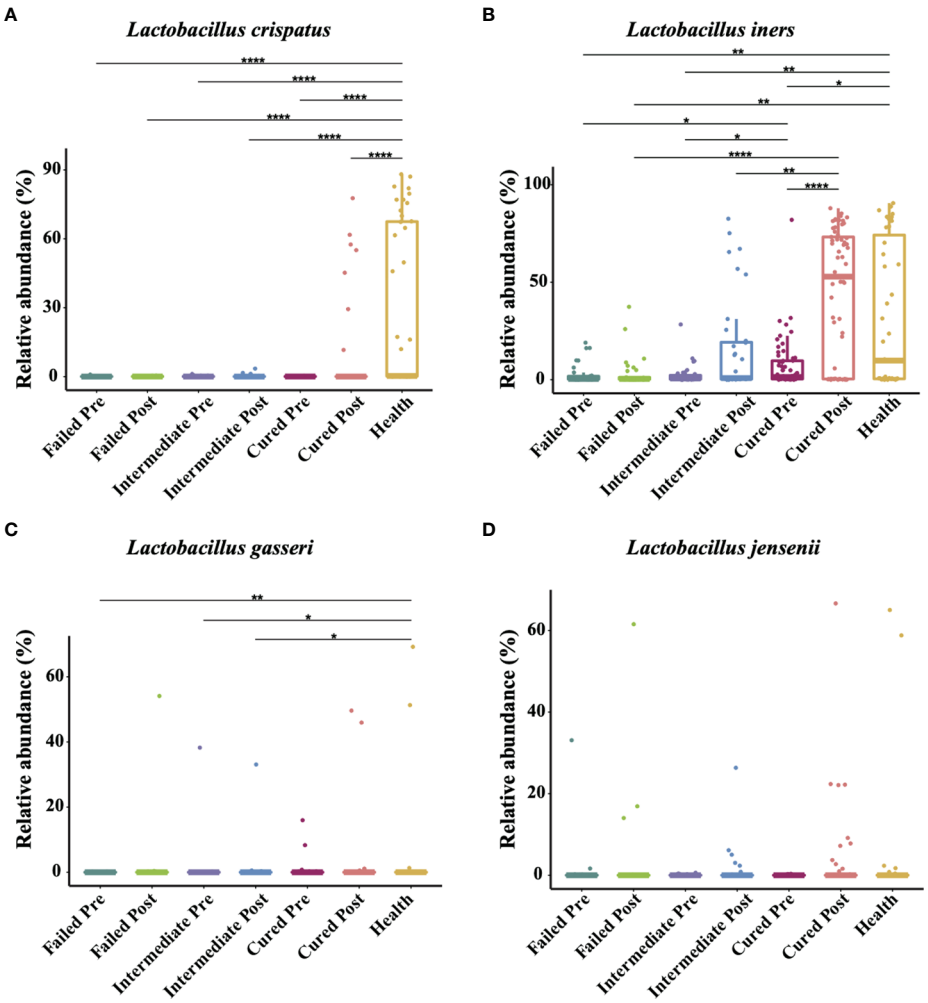

FIGURE 4
Comparison of four *Lactobacillus* species abundance in different groups. Intergroup comparison of the relative abundance of the four most abundant *Lactobacillus* species: *L. crispatus* **(A)**, *L. iners* **(B)**, *L. gasseri* **(C)** and *L. jensenii* **(D)**. Only statistically significant *P* value that has clinical meaning is marked in the graph. Significance is exhibited as: *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; Wilcoxon test for pairwise comparison between pre- and posttreatment and Kruskal-Wallis test for comparisons among different groups.

iners is higher at the time of treatment, its antimicrobial abilities might facilitate the therapy of BV, as it is resistant to metronidazole and able to scavenge pathogens simultaneously. Furthermore, considering that *L. iners* is capable of synthesizing L-lactic acid and a small amount of D-lactic acid (Vaneechoutte, 2017), we hypothesized that the restoration of *L. iners* after treatment might be crucial for the recovery of other *Lactobacillus* by maintaining an acidic environment and countering the growth of BV-associated bacteria.

This study is the first attempt to describe the distribution of *Gardnerella* genomospecies in Chinese women to determine its relationship with the clinical outcomes of BV patients. Moreover, we also validated the inhibitory effect of *L. iners* against *G. vaginalis* and *F. vaginae* through coculture experiments. Though with limited sample size and restricted experimental conditions, our conclusion could be more general with more incorporated participants. Nonetheless, our *in vitro* test is only preliminary in explaining the correlation of *L. iners* with BV clinical outcomes. In future studies, more experiments and animal models are needed to reveal the mechanism underneath the relation between *L. iners* and BV clinical outcomes and more participants are required to better represent the *Gardnerella* genomospecies distribution in China.

Conclusion

Our research found seven *Gardnerella* genomospecies and revealed that Chinese women and cured patients possessed higher GS03 and *L. iners* pretreatment and validated the

inhibitory effect of *L. iners* against the growth of *Gardnerella vaginalis* and *Fannyhessae vaginae*. Finally, we suggest gynecologists have a better understanding of the vaginal microbiota of BV patients pretreatment to improve their overall health and that *L. iners* might become an innovative biomarker for BV treatment outcomes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://nmdc.cn/resource/attachment/detail/NMDCX0000148.

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee of Peking University First Hospital (2020[083]-001). The patients/participants provided their written informed consent to participate in this study.

Author contributions

BX and JW designed the project. BX collected samples. RZ and JL ran the experiment procedure. All authors participated in data analysis, writing and discussing the contents of this article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1033431/full#supplementary-material

References

Abou Chacra, L., Fenollar, F., and Diop, K. (2021). Bacterial vaginosis: What do we currently know? *Front. Cell Infect. Microbiol.* 11, 672429. doi: 10.3389/fcimb.2021.672429

Amsel R Fau - Totten, P. A., Totten Pa Fau - Spiegel, C. A., Spiegel Ca Fau - Chen, K. C., Chen Kc Fau - Eschenbach, D., Eschenbach, D., Fau - Holmes, K. K., et al. (1983). Nonspecific vaginitis. *Diagn. Criteria Microb. Epidemiol. Associations.* 74 (1), 14–22. doi: 10.1016/0002-9343(83)91112-9

Anderson, M. J. A. W., T.J. (2003). Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. *Ecology* 84, 15. doi: 10.1890/0012-9658(2003)084[0511:CAOPCA]2.0.CO;2

Armstrong, E., Hemmerling, A., Miller, S., Burke, K. E., Newmann, S. J., Morris, S. R., et al. (2022). Metronidazole treatment rapidly reduces genital inflammation through effects on bacterial vaginosis-associated bacteria rather than lactobacilli. *J. Clin. Invest.* 132 (6), e152930. doi: 10.1172/JCI152930

Bloom, S. M., Mafunda, N. A., Woolston, B. M., Hayward, M. R., Frempong, J. F., Abai, A. B., et al. (2022). Cysteine dependence of lactobacillus iners is a potential therapeutic target for vaginal microbiota modulation. *Nat. Microbiol.* 7, 434–450. doi: 10.1038/s41564-022-01070-7

Bostwick, D. G., Woody, J., Hunt, C., and Budd, W. (2016). Antimicrobial resistance genes and modelling of treatment failure in bacterial vaginosis: clinical study of 289 symptomatic women. *J. Med. Microbiol.* 65, 377–386. doi: 10.1099/jmm.0.000236

Deng, Z. L., Gottschick, C., Bhuju, S., Masur, C., Abels, C., and Wagner-Dobler, I. (2018). Metatranscriptome analysis of the vaginal microbiota reveals potential mechanisms for protection against metronidazole in bacterial vaginosis. *mSphere* 3 (3), e00262–18. doi: 10.1128/mSphereDirect.00262-18

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., et al. (2016). Population-level analysis of gut microbiome variation. *Science* 352, 560–564. doi: 10.1126/science.aad3503

Faught, B. M., and Reyes, S. (2019). Characterization and treatment of recurrent bacterial vaginosis. *J. Womens Health (Larchmt)* 28, 1218–1226. doi: 10.1089/jwh.2018.7383

Ferreira, C. S. T., Marconi, C., Parada, C., Ravel, J., and Da Silva, M. G. (2021). Sialidase activity in the cervicovaginal fluid is associated with changes in bacterial components of lactobacillus-deprived microbiota. *Front. Cell Infect. Microbiol.* 11, 813520. doi: 10.3389/fcimb.2021.813520

France, M., Alizadeh, M., Brown, S., Ma, B., and Ravel, J. (2022). Towards a deeper understanding of the vaginal microbiota. *Nat. Microbiol.* 7, 367–378. doi: 10.1038/s41564-022-01083-2

France, M. T., Fu, L., Rutt, L., Yang, H., Humphrys, M. S., Narina, S., et al. (2022). Insight into the ecology of vaginal bacteria through integrative analyses of metagenomic and metatranscriptomic data. *Genome Biol.* 23, 66. doi: 10.1186/s13059-022-02635-9

France, M. T., Mendes-Soares, H., and Forney, L. J. (2016). Genomic comparisons of lactobacillus crispatus and lactobacillus iners reveal potential ecological drivers of community composition in the vagina. *Appl. Environ. Microbiol.* 82, 7063–7073. doi: 10.1128/AEM.02385-16

France, M. A.-O., Rutt, L., Narina, S., Arbaugh, S., Mccomb, E. A.-O., Humphrys, M. S., et al. (2020). Complete genome sequences of six lactobacillus iners strains isolated from the human vagina. *LID - 10.1128/MRA.00234-20 [doi] LID* -, e00234–e00220. doi: 10.1128/MRA.00234-20

Hardy, L., Jespers, V., Van Den Bulck, M., Buyze, J., Mwambarangwe, L., Musengamana, V., et al. (2017). The presence of the putative gardnerella vaginalis sialidase a gene in vaginal specimens is associated with bacterial vaginosis biofilm. *PloS One* 12, e0172522. doi: 10.1371/journal.pone.0172522

Harwich, M. D.Jr, Alves, J. M., Buck, G. A., Strauss, J. F.3rd, Patterson, J. L., Oki, A. T., et al. (2010). Drawing the line between commensal and pathogenic gardnerella vaginalis through genome analysis and virulence studies. *BMC Genomics* 11, 375. doi: 10.1186/1471-2164-11-375

Hilbert, D. W., Schuyler, J. A., Adelson, M. E., Mordechai, E., Sobel, J. D., and Gygax, S. E. (2017). Gardnerella vaginalis population dynamics in bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 1269–1278. doi: 10.1007/s10096-017-2933-8

Hill, J. A.-O., and Albert, A. A.-O. (2019). Resolution and cooccurrence patterns of gardnerella leopoldii, g. swidsinskii, g. piotii and g. vaginalis within the vaginal microbiome. *LID* -, e00532-19. doi: 10.1128/IAI.00532-19

Hill, J. A.-O., and Albert, A. A.-O. (2020). Resolution and cooccurrence patterns of gardnerella leopoldii, g. swidsinskii, g. piotii and g. vaginalis within the vaginal microbiome. *LID* -, e00532-19. doi: 10.1128/IAI.00532-19

Janulaitiene, M., Gegzna, V., Baranauskiene, L., Bulavaite, A., Simanavicius, M., and Pleckaityte, M. (2018). Phenotypic characterization of gardnerella vaginalis subgroups suggests differences in their virulence potential. *PloS One* 13, e0200625. doi: 10.1371/journal.pone.0200625

Jung, H. S., Ehlers, M. M., Lombaard, H., Redelinghuys, M. J., and Kock, M. M. (2017). Etiology of bacterial vaginosis and polymicrobial biofilm formation. *Crit. Rev. Microbiol.* 43, 651–667. doi: 10.1080/1040841X.2017.1291579

Kenyon, C., Colebunders, R., and Crucitti, T. (2013). The global epidemiology of bacterial vaginosis: a systematic review. *Am. J. Obstet. Gynecol.* 209, 505–523. doi: 10.1016/j.ajog.2013.05.006

Khan, S., Vancuren, S. J., and Hill, J. E. (2021). A generalist lifestyle allows rare gardnerella spp. to persist at low levels in the vaginal microbiome. *Microb. Ecol.* 82, 1048–1060. doi: 10.1007/s00248-020-01643-1

King, C. C., Jamieson, D. J., Wiener, J., Cu-Uvin, S., Klein, R. S., Rompalo, A. M., et al. (2011). Bacterial vaginosis and the natural history of human papillomavirus. *Infect. Dis. Obstet. Gynecol.* 2011, 319460. doi: 10.1155/2011/319460

Lee, C. Y., Cheu, R. K., Lemke, M. M., Gustin, A. T., France, M. T., Hampel, B., et al. (2020). Quantitative modeling predicts mechanistic links between pre-treatment microbiome composition and metronidazole efficacy in bacterial vaginosis. *Nat. Commun.* 11 (1), 6147–6158. doi: 10.1038/s41467-020-19880-w

Lehtoranta, L., Hibberd, A. A., Reimari, J., Junnila, J., Yeung, N., Maukonen, J., et al. (2020). Recovery of vaginal microbiota after standard treatment for bacterial vaginosis infection: An observational study. *Microorganisms* 8 (6), 875–892. doi: 10.3390/microorganisms8060875

Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mohankumar, B., Shandil, R. K., Narayanan, S., and Krishnan, U. M. (2022). Vaginosis: Advances in new therapeutic development and microbiome restoration. *Microb. Pathog.* 168, 105606. doi: 10.1016/j.micpath.2022.105606

Morrill, S., Gilbert, N. M., and Lewis, A. L. (2020). Gardnerella vaginalis as a cause of bacterial vaginosis: Appraisal of the evidence from *in vivo* models. *Front. Cell Infect. Microbiol.* 10, 168. doi: 10.3389/fcimb.2020.00168

Munoz-Barreno, A., Cabezas-Mera, F., Tejera, E., and Machado, A. (2021). Comparative effectiveness of treatments for bacterial vaginosis: A network meta-analysis. *Antibiotics (Basel)* 10 (8), 978–993. doi: 10.3390/antibiotics10080978

Muzny, C. A., Blanchard, E., Taylor, C. M., Aaron, K. J., Talluri, R., Griswold, M. E., et al. (2018). Identification of key bacteria involved in the induction of incident bacterial vaginosis: A prospective study. *J. Infect. Dis.* 218, 966–978. doi: 10.1093/infdis/jiy243

Muzny, C. A., Taylor, C. M., Swords, W. E., Tamhane, A., Chattopadhyay, D., Cerca, N., et al. (2019). An updated conceptual model on the pathogenesis of bacterial vaginosis. *J. Infect. Dis.* 220, 1399–1405. doi: 10.1093/infdis/jiz342

Novak, J., Ravel, J., Ma, B., Ferreira, C. S. T., Tristao, A. D. R., Silva, M. G., et al. (2022). Characteristics associated with lactobacillus iners-dominated vaginal microbiota. *Sex Transm. Infect.* 98, 353–359. doi: 10.1136/sextrans-2020-054824

Nugent, R. P., Krohn Ma Fau - Hillier, ,. S. L., and Hillier, S. L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol.* 29 (2), 297–301. doi: 10.1128/jcm.29.2.297-301.1991

Numanović, F., Hukić M Fau - Nurkić, M., Nurkić M Fau - Gegić, M., Gegić M Fau - Delibegović, Z., Delibegović Z Fau - Imamović, A., and Imamović A Fau - Pasić, S. (2008). Importance of isolation and biotypization of gardnerella vaginalis in diagnosis of bacterial vaginosis. *Bosn J Basic Med Sci.* 8 (3), 270–276. doi: 10.17305/bjbms.2008.2932

Onderdonk, A. B., Delaney, M. L., and Fichorova, R. N. (2016). The human microbiome during bacterial vaginosis. *Clin. Microbiol. Rev.* 29, 223–238. doi: 10.1128/CMR.00075-15

Pacha-Herrera, D., Erazo-Garcia, M. P., Cueva, D. F., Orellana, M., Borja-Serrano, P., Arboleda, C., et al. (2022). Clustering analysis of the multi-microbial consortium by lactobacillus species against vaginal dysbiosis among Ecuadorian women. *Front. Cell Infect. Microbiol.* 12, 863208. doi: 10.3389/fcimb.2022.863208

Pleckaityte, M. (2019). Cholesterol-dependent cytolysins produced by vaginal bacteria: Certainties and controversies. *Front. Cell Infect. Microbiol.* 9, 452. doi: 10.3389/fcimb.2019.00452

Qin, H., and Xiao, B. (2022). Research progress on the correlation between gardnerella typing and bacterial vaginosis. *Front. Cell Infect. Microbiol.* 12, 858155. doi: 10.3389/fcimb.2022.858155

Ragaliauskas, T., Pleckaityte, M., Jankunec, M., Labanauskas, L., Baranauskiene, L., and Valincius, G. (2019). Inerolysin and vaginolysin, the cytolysins implicated in vaginal dysbiosis, differently impair molecular integrity of phospholipid membranes. *Sci. Rep.* 9, 10606. doi: 10.1038/s41598-019-47043-5

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., Mcculle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U.S.A.* 108 (Suppl 1), 4680–4687. doi: 10.1073/pnas.1002611107

Ravel, J., Moreno, I., and Simon, C. (2021). Bacterial vaginosis and its association with infertility, endometritis, and pelvic inflammatory disease. *Am. J. Obstet. Gynecol.* 224, 251–257. doi: 10.1016/j.ajog.2020.10.019

Rosca, A. S., Castro, J., Sousa, L. G. V., Franca, A., Vaneechoutte, M., and Cerca, N. (2022). *In vitro* interactions within a biofilm containing three species found in bacterial vaginosis (BV) support the higher antimicrobial tolerance associated with BV recurrence. *J. Antimicrob. Chemother.* 77, 2183–2190. doi: 10.1093/jac/dkac155

Santiago, G. L., Deschaght, P., El Aila, N., Kiama, T. N., Verstraelen, H., Jefferson, K. K., et al. (2011). Gardnerella vaginalis comprises three distinct genotypes of which only two produce sialidase. *Am. J. Obstet. Gynecol.* 204, 450 e1–e7.

Schellenberg, J. J., Paramel Jayaprakash, T., Withana Gamage, N., Patterson, M. H., Vaneechoutte, M., and Hill, J. E. (2016). Gardnerella vaginalis subgroups defined by cpn60 sequencing and sialidase activity in isolates from Canada, Belgium and Kenya. *PloS One* 11, e0146510. doi: 10.1371/journal.pone.0146510

Schellenberg, J. J., Patterson, M. H., and Hill, J. E. (2017). Gardnerella vaginalis diversity and ecology in relation to vaginal symptoms. *Res. Microbiol.* 168, 837–844. doi: 10.1016/j.resmic.2017.02.011

Schwebke, J. R., Muzny, C. A., and Josey, W. E. (2014). Role of gardnerella vaginalis in the pathogenesis of bacterial vaginosis: a conceptual model. *J. Infect. Dis.* 210, 338–343. doi: 10.1093/infdis/jiu089

Shipitsyna, E., Krysanova, A., Khayrullina, G., Shalepo, K., Savicheva, A., Guschin, A., et al. (2019). Quantitation of all four gardnerella vaginalis clades detects abnormal vaginal microbiota characteristic of bacterial vaginosis more accurately than putative g. vaginalis sialidase a gene count. *Mol. Diagn. Ther.* 23, 139–147. doi: 10.1007/s40291-019-00382-5

Somerfield, P. J., Clarke, K. R., and Gorley, R. N. (2021). Analysis of similarities (ANOSIM) for 2-way layouts using a generalised ANOSIM statistic, with comparative notes on permutational multivariate analysis of variance (PERMANOVA). *Austral Ecol.* 46, 16. doi: 10.1111/aec.13059

Srinivasan, S., Hoffman, N. G., Morgan, M. T., Matsen, F. A., Fiedler, T. L., Hall, R. W., et al. (2012). Bacterial communities in women with bacterial vaginosis: high

resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PloS One* 7, e37818. doi: 10.1371/journal.pone.0037818

Turner, E., Sobel, J. D., and Akins, R. A. (2021). Prognosis of recurrent bacterial vaginosis based on longitudinal changes in abundance of lactobacillus and specific species of gardnerella. *PloS One* 16, e0256445. doi: 10.1371/journal.pone.0256445

Vaneechoutte, M. (2017). Lactobacillus iners, the unusual suspect. *Res. Microbiol.* 168, 826–836. doi: 10.1016/j.resmic.2017.09.003

Vaneechoutte, M., Guschin, A., Van Simaey, L., Gansemans, Y., Van Nieuwerburgh, F., and Cools, P. (2019). Emended description of gardnerella vaginalis and description of gardnerella leopoldii sp. nov., gardnerella piotii sp. nov. and gardnerella swidsinskii sp. nov., with delineation of 13 genomic species within the genus gardnerella. *Int. J. Syst. Evol. Microbiol.* 69, 679–687. doi: 10.1099/ijsem.0.003200

Verwijs, M. C., Agaba, S. K., Darby, A. C., and Van De Wijgert, J. (2020). Impact of oral metronidazole treatment on the vaginal microbiota and correlates of treatment failure. *Am. J. Obstet. Gynecol.* 222, 157.e1–157 e13. doi: 10.1016/j.ajog.2019.08.008

Vestby, L. K., Gronseth, T., Simm, R., and Nesse, L. L. (2020). Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics (Basel)* 9 (2), 52–96. doi: 10.3390/antibiotics9020059

Vodstrcil, L. A., Twin, J., Garland, S. M., Fairley, C. K., Hocking, J. S., Law, M. G., et al. (2017). The influence of sexual activity on the vaginal microbiota and gardnerella vaginalis clade diversity in young women. *PloS One* 12, e0171856. doi: 10.1371/journal.pone.0171856

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Zheng, N., Guo, R., Wang, J., Zhou, W., and Ling, Z. (2021). Contribution of lactobacillus iners to vaginal health and diseases: A systematic review. *Front. Cell Infect. Microbiol.* 11, 792787. doi: 10.3389/fcimb.2021.792787

Zhou, X., Hansmann, M. A., Davis, C. C., Suzuki, H., Brown, C. J., Schutte, U., et al. (2010). The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. *FEMS Immunol. Med. Microbiol.* 58, 169–181. doi: 10.1111/j.1574-695X.2009.00618.x

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Zuo Zhang,
Nanchang University, China
Hao Zhang,
University of Pennsylvania, United States

*CORRESPONDENCE
Jianqiang Chen
93685100@qq.com
Qiaoxin Zhang
qiaoxinzhang@126.com

†These authors have contributed equally to this work and share the first authorship

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Roles of vaginal flora in human papillomavirus infection, virus persistence and clearance

Mi Zeng[1†], Xin Li[1†], Xiaoyang Jiao[1,2], Xiaochun Cai[3], Fen Yao[2,4], Shaomin Xu[3], Xiaoshan Huang[5], Qiaoxin Zhang[6*] and Jianqiang Chen[6*]

[1]Department of Cell Biology and Genetics, Shantou University Medical College, Shantou, Guangdong, China, [2]Guangdong Provincial Key Laboratory of Infectious Diseases and Molecular Immunopathology, Shantou, Guangdong, China, [3]Department of Gynecology and Obstetrics, Chenghai District People's Hospital, Shantou, Guangdong, China, [4]Department of Pharmacology, Shantou University Medical College, Shantou, Guangdong, China, [5]Longhu Hospital, The First Affiliated Hospital of Shantou University Medical College, Shantou, China, [6]The First Affiliated Hospital of Shantou University Medical College, Shantou, China

Vaginal flora plays a vital role in human papillomavirus (HPV) infection and progression to cancer. To reveal a role of the vaginal flora in HPV persistence and clearance, 90 patients with HPV infection and 45 healthy individuals were enrolled in this study and their vaginal flora were analyzed. Women with HPV infection were treated with *Lactobacillus* in the vaginal environment as a supplement to interferon therapy. Our results indicated that patients with high risk HPV (Hr-HPV) 16/18 infection had a significantly higher alpha diversity compared with the healthy control ($p < 0.01$), while there was no significant difference between the non-Hr-HPV16/18 group and the controls ($p > 0.05$). Patients with multiple HPV infection had insignificantly higher alpha diversity compared with single HPV infection ($p > 0.05$). The vaginal flora of patients with HPV infection exhibited different compositions when compared to the healthy controls. The dominant bacteria with the highest prevalence in HPV-positive group were *Lactobacillus iners* (n = 49, 54.44%), and the top 3 dominant bacteria in the HPV-persistent group were *Lactobacillus iners* (n = 34, 53.13%), *Sneathia amnii* (n = 9, 14.06%), and *Lactobacillus delbrueckii* (n = 3, 4.69%). Patients with HPV clearance had significantly lower alpha diversity, and the flora pattern was also different between groups displaying HPV clearance vs. persistence. The patients with persistent HPV infection had significantly higher levels of Bacteroidaceae, Erysipelotrichaceae, Helicobacteraceae, Neisseriaceae, Streptococcaceae (family level), and *Fusobacterium*, *Bacteroides*, *Neisseria*, and *Helicobacter* (genus level) than patients who had cleared HPV ($p < 0.05$).

Importance: Our study revealed differences in vaginal flora patterns are associated with HPV persistence and its clearance. Interferon plus probiotics can greatly improve virus clearance in some patients. Distinguishing bacterial features associated with HPV clearance in patients would be helpful for early intervention and reverse persistent infection.

KEYWORDS

human papillomavirus, vaginal microbiota, lactobacillus, 16S ribosomal DNA sequencing, probiotics

Introduction

Human papillomavirus (HPV) infection is one of the most common sexually transmitted infections, and is also the leading cause of cervical cancers (Lehtinen et al., 2019). Up to now, 396 distinct HPV subtypes have been reported (Bzhalava et al., 2014). Genital HPVs can be subdivided into high- and low-risk types, with 13 being identified as high-risk HPVs (Hr-HPVs) (Walboomers et al., 1999), and the two most common cervical Hr-HPVs are HPV16 and 18. Most low-risk types of HPV infections resolve over time. However, persistent cervical Hr-HPV infections play a crucial role in the development of cervical cancer (Schiffman et al., 2016). HPV infection is actively involved in cervical epithelial transformation (Wilkinson et al., 2015; Curty et al., 2017). Although approximately 70% of cervical cancer cases worldwide are caused by HR-HPV (Clifford et al., 2003; Oliveira and Schirger, 2003; Du et al., 2011; Chan et al., 2019), not all people with HPV infection actually end up developing cancer and only a small percentage of Hr-HPV infections develop into cervical cancer, indicating that virus infection is not sufficient for cancer development, additional factors may involve in HPV inducing cervical cancer (Usyk et al., 2020).

Recent studies have shown factors, including integrity of epithelial surface, mucosal secretions, immune regulation, and the local microbiota, play a part in the development of HPV infection to cancer (Pyeon et al., 2009; Fernandes et al., 2015; Schiffman et al., 2016). More than 200 bacterial species comprise the vaginal flora of healthy women, which are mainly dominated by one of the four most prevalent *Lactobacillus* species: *Lactobacillus crispatus* (*L. crispatus*), *Lactobacillus iners* (*L. iners*), *Lactobacillus gasseri* (*L. gasseri*), and *Lactobacillus jensenii* (*L. jensenii*). *Lactobacillus* spp. form barriers against colonization of bacterial vaginosis (BV)-associated bacteria by maintaining a low pH (Mastromarino et al., 2014; Breshears et al., 2015). It is essential for maintaining cervical epithelial barrier function which inhibits infection of basal keratinocytes by HPV (Borgdorff et al., 2016). BV is also connected with an increase in the production of epithelial lining-degrading enzymes that can allow the initiation of HPV infection (Kabuki et al., 1997; Gillet et al., 2012; Stoyancheva et al., 2014). Therefore, vaginal *Lactobacillus* spp. play a significant impact in the persistence or regression of the virus and subsequent disease (Petrova et al., 2013; Brotman et al., 2014; DiGiulio et al., 2015; Mitra et al., 2016). Invasive cervical cancer patients exhibit decreased *Lactobacillus* spp., increased *Fusobacterium* spp., and increased overall bacterial diversity and richness (Lin et al., 2020). *Fusobacterium* predominance is more prevalent in individuals with invasive cervical cancer, where it is found to be related with elevated levels of IL-4 and transforming growth factor (TGF)-β1 mRNA, indicating its immunosuppressive effect in the microenvironment of the invasive cervical cancer (Audirac-Chalifour et al., 2016). Microbiota dysbacteriosis might increase the apoptosis of cancer cells or might activate immunosuppressive cells, such as dendritic and Treg cells, and cytokines (Nami et al., 2014; Nami et al., 2014; Eslami et al., 2016; Wang et al., 2018). Therefore, dysbacteriosis has lately been associated with cancer progression and treatment responses (Chang and Parsonnet, 2010).

The function of the vaginal flora in HPV-driven disease has been intensively explored. A previous study has found changes in the female genital tract microbial flora to be related to HPV infection and cervical cancer (Nieves-Ramírez et al., 2021). *Sneathia* and *Prevotella* enrichment is highly related to HPV infection and contributes to HPV persistent infection (Di Paola et al., 2017; Łaniewski et al., 2018; Brusselaers et al., 2019). Both BV and cervical intraepithelial neoplasia (CIN) show a similar characteristics of vaginal flora, which present a decrease in *Lactobacilli* abundance, increased diversity and an increase in the predominance of abnormal anaerobic bacteria (Gillet et al., 2012). Disruption of protective microbiota colonization can lead to a weakening of defense mechanisms. Although the field of microbiome about HPV-driven cancers is emerging rapidly, with most studies focusing on characterizing bacterial profiles, a possible association between vaginal flora composition and HPV clearance or progression to cervical dysplasia and cancer has yet to be shown (Mitra et al., 2016; Shannon et al., 2017; Godoy-Vitorino et al., 2018; Lin et al., 2020; Norenhag et al., 2020; Mitra et al., 2020). Given the part of low *Lactobacillus* cases, more detailed community state types (CSTs) of bacteria in addition to *Lactobacilli* might be helpful for vaginal microbiome studies (Cheng et al., 2020).

It has been reported that a vaginal flora dominated by non-*Lactobacillus* species is connected with the risk of HPV infection and persistence (Lee et al., 2013; Mitra et al., 2016; Shannon et al., 2017; Norenhag et al., 2020). Some *Lactobacillus* species like *L. gasseri*, might be helpful for the clearance of HPV (Brotman et al., 2014; Brusselaers et al., 2019). The probiotic can alter the tumor microenvironment. When *Lactobacillus casei*-containing probiotics were administered to HPV-positive women, enhanced HPV clearance were observed (Verhoeven et al., 2013). Oral probiotics might be helpful to preserve normal vaginal flora during antibiotic therapy (Macklaim et al., 2015), but its efficacy varies widely, and may be influenced by many factors, including interruption from the gut local microenvironment and colonization of bacteria. At the very beginning, limited data are available (Li et al., 2020). Probiotics directly applied to the vaginal environment may play a more direct role in vaginal flora, but little information has been obtained in this field. A deeper understanding of vaginal flora will eventually aid in the development of practical and low-cost treatments to reduce the HPV infection (Li et al., 2020). Given the lack of research in this area, more studies are required to elucidate the effect of probiotic therapy on specific microbiota in patients with HPV infection. In this study, vaginal probiotics (mainly *lactobacilli*) were used to treat the patients with HPV infection, and the entire composition of vaginal microbiota was studied. We also discuss the impact of vaginal flora on HPV clearance.

Methods

Patients

A participant was eligible if she (a) was 18-60 years old without HPV vaccination, (b) had not undergone a gynecological reproductive surgery such as cervical conization, hysterectomy, appendectomy, hysteroscopy, etc, (c) had no vaginal flushing and had abstained from sex for at least 72 h, (d) had no history of vaginal medication within 3 days, and no systematic use of antibiotics or antifungal drugs, probiotics, antibiotics or glucocorticoids within 1 month, and (e) was HPV-positive upon initial screening. The 21 HPV GenoArray Diagnostic Kit (Chaozhou Hybribio Biochemistry Ltd, China) was used to conducted HPV typing. Genotypes of 21 HPV genotypes (6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68 and CP8304 (81)) were detected. Healthy women were enrolled from the physical examination center of Chenghai district people's hospital; all were HPV-negative. Exclusion criteria: (a) had a vaginal lavage or had sexual activity within 72 hours, (b) used probiotic bacteria, antibiotics, or corticosteroids within the past 30 days, (c) with cancer, diabetes, autoimmune diseases and other serious diseases that may affect the results of this study, (d) was pregnant.

A total of 135 participants were included on the basis of the inclusion and exclusion criteria. All procedures for this study were approved by the Research Ethics Committee of the First Affiliated Hospital of Shantou University Medical College (No. 201561).

Sample collection, DNA extraction, and 16S sequencing

Cervical specimens were collected from female patients between January 2016 and June 2018.

Genomic DNA was extracted from the samples by using the CTAB (Cetyltrimethylammonium Bromide) method. After the detection of purity and concentration of genomic DNA, using genomic DNA diluted with sterile water to 1 ng/μL, specific primers with barcode, New England Biolabs Phusion® High Fidelity PCR Master Mix with GC Buffer (New England Biolabs, USA) and Phusion® High-Fidelity DNA polymerase (New England Biolabs, USA) were used for PCR. After mixing in equal amounts according to the PCR product concentration, electrophoresis purification was performed on a 1× TAE 2% agarose gel, shearing to obtain the band of interest, and the sheared target bands were recovered using a DNA purification kit (DP214, Tiangen, China). The construction of library was performed using Ion Plus Fragment Library Kit 48 (Thermo Fisher, USA), which was sequenced using an Ion S5™XL (Thermo Fisher, USA) after Qubit quantification and library detection.

Bioinformatics analyses

Low-quality data was removed by using Cutadapt (V1.9.1) (Langille et al., 2013). Then barcode and primer sequences were trimmed. Clean reads were obtained after detecting and removing chimeric sequences (Rognes et al., 2016) from raw data by using VSEARCH (Martin, 2011). All clean reads of all samples were clustered as Operational Taxonomic Units (OTUs) using the UPARSE algorithm (UPARSE v7.0.1001) (Haas et al., 2011) by default with 97% identity. The annotation of OTUs representative sequences were performed by using Mothur method and SSUrRNA database (Wang et al., 2007) of SILVA132 (Edgar, 2013) (threshold was set at 0.8~1) obtain species information and species abundance at each taxonomic level. MUSCLE (Quast et al., 2013) (Version 3.8.31), software was used to do fast sequence alignment and then the process of homogenization was conducted. ALPHA diversity indices and the UniFrac distance were calculated by using QIIME software (Version 1.9.1) (Caporaso et al., 2010).

Statistical analyses

Alpha diversities were visualized in the box plot using the package "ggplot2" in R software (Version 4.3.0). Principal co-ordinates analysis (PCoA) was conducted on basis of the unweighted UniFrac distance matrix using "vegan", "Parseq", and "ggplot2" packages. PERMANOVA analysis was conducted using the "vegan" package. Linear discriminant analysis Effect Size (LEfSe) analysis was done by using LEfSe software, with the default value of the linear discriminant analysis (LDA) score being 2. The Chord diagram was performed using the "circlize" package in R. Kruskal-Wallis tests were employed to analyze differences in microbial α diversity among multiple groups. If $p < 0.05$, Dunn's Test was used to perform pairwise comparisons between each independent group. Measurement data for demographic and clinical characteristics were analyzed using the Kruskal-Wallis test, while the ordinal categorical variable was analyzed using the Wilcoxon rank-sum test. The association between HPV and age, cleaning degree of the vagina, menopause, contraception, non-menstrual bleeding, the number of pregnancies, births, miscarriages, and the number of white blood cells were analyzed by Spearman rank correlation analysis in SPSS 26.0.

Results

Patient's characteristics

In total, 135 participants were included in the research (Figure S1), including 45 normal controls and 90 patients with HPV infection, in which the HPV subtypes with the highest

frequencies were HPV16 (22.22%, n = 20), HPV51 (16.67%, n = 15), HPV53 (16.67%, n = 15), and HPV52 (15.56%, n = 14). The samples were divided into two groups based on whether the infected HPV type was HPV16/18 (Hr-HPV16/18, n = 28) or was non-HPV16/18 (non-Hr-HPV16/18, n = 62). The morbidity of HPV16 was 71.43% (n = 20), and for HPV18 was 28.57% (n = 8) in the Hr-HPV16/18 group, while in the non-Hr-HPV16/18 group, the main types were HPV51, HPV52, and HPV53, and their morbidities were 22.58% (n = 14), 19.35% (n = 12), and 20.97% (n = 13), respectively (Figure 1). The difference in age among the three groups (controls, 36 (IQR = 17); Hr-HPV16/18, 37 (IQR = 18); non-Hr-HPV16/18, 41.5 (IQR = 10.75), $p > 0.05$) was not statistically significant. Only a small number of women in the three groups were in menopause; the ratio of women in menopause was 13.33% (n = 6) in the controls, 7.14% (n = 2) in Hr-HPV16/18, and 17.74% (n = 11) in non-Hr-HPV16/18 patients, respectively. But patients with non-menstrual bleeding had significant differences among the Hr-HPV16/18, non-Hr-HPV16/18, and the controls ($p < 0.01$). No positive cases of BV were found, but some cases (n = 14) have tested positive for Ureaplasma urealyticum, M. hominis, and Chlamydia trachomatis, and only one positive case of trichomoniasis. Other parameters, including squamous intraepithelial disease (SIL) grade, menopause, and IUD/tubal ligation/condom, the number of pregnancies, births, miscarriages, and white blood cells, were measured and showed no significant difference ($p > 0.05$). Detailed demographics and clinical characteristics of participants were showed in Table 1.

Vaginal bacterial diversity in patients with HPV and non-HPV infection

High-quality classifiable 16S ribosomal DNA sequences were acquired, with 77,391 clean reads per sample. Bacterial communities and their alpha diversity were measured. Our results showed that there was a significantly higher observed species, and ACE and Chao1 scores in the Hr-HPV16/18 groups than those in controls (Figure 2), while there was no significant difference in Shannon and Simpson indices between the two groups ($p > 0.05$), and there was no significant difference between the non-Hr-HPV16/18 groups and the controls ($p > 0.05$). These results show a higher microbial diversity in the Hr-HPV16/18 groups. No clear separation of samples between the HPV-uninfected and infected groups was showed in PCoA analysis, indicating there were no significant similarity differences in microbial composition among the three groups (Figure 3).

Comparison of bacteria at the phylum, genus, and species levels between the HPV-infected group and controls

In vaginal flora, Firmicutes was the most predominant phylum in the healthy individuals and HPV-infected patients, followed by Proteobacteria, Actinobacteria, Fusobacteria, and Bacteroidetes. There was no significant difference between the HPV-infected group and control. Then, we characterized the differences in vaginal flora among the three groups at the genus level. The top 10 bacteria in the three groups (Hr-HPV16/18 vs. non-Hr-HPV16/18 vs. controls) were: *Lactobacillus* spp. (46.47% vs. 43.46% vs. 39.04%, $p > 0.05$); *Gardnerella* spp. (6.60% vs. 12.69% vs. 17.07%, $p > 0.05$); *Sneathia* spp. (6.15% vs. 7.59% vs. 5.94%, $p > 0.05$); *Prevotella* spp. (2.61% vs. 4.73% vs. 4.17%, $p > 0.05$); *Klebsiella* spp. (2.83% vs. 2.93% vs. 3.62%, $p > 0.05$); *Streptococcus* spp. (3.38% vs. 1.48% vs. 3.94%, $p > 0.05$); *Enterococcus* spp. (4.56% vs.1.49% vs. 1.48%, $p > 0.05$); *Staphylococcus* spp. (3.43% vs. 0.83% vs. 1.39%, $p > 0.05$); *Atopobium* spp. (0.59% vs. 1.51%, vs. 0.89%, $p < 0.05$), and *Sphingomonas* spp. (3.36%, vs. 0.01% vs.0.02%, $p < 0.05$) (Figures 4, 5).

At the bacterial species level, the top 10 species in three groups (Hr-HPV16/18 vs. non-Hr-HPV16/18 vs. controls) were: *L. iners* (26.29% vs. 30.16% vs. 23.27%, $p > 0.05$); *Sneathia amnii* (*S. amnii*) (5.08% vs. 4.69% vs. 2.11%, $p < 0.05$); *Enterococcus faecalis* (*E. faecalis*) (3.37% vs. 1.34%, vs. 1.38%, $p > 0.05$); *Sneathia sanguinegens* (*S. sanguinegens*) (0.46% vs. 1.29% vs. 1.56%, $p > 0.05$); *L. gasseri* (0.09% vs. 1.33% vs. 1.72%, $p < 0.05$); *Staphylococcus haemolyticus* (*S. haemolyticus*) (2.38% vs. 0.71% vs. 0.91%, $p > 0.05$); *Atopobium vaginae* (*A. vaginae*) (0.59% vs. 1.51% vs. 0.88%, $p < 0.05$); *Prevotella amnii* (*P. amnii*) (0.06% vs. 1.34% vs.1.34%, p>0.05); *Acinetobacter nosocomialis* (*A. nosocomialis*) (0.02%; vs. 0.67% vs. 2.08%, $p > 0.05$), and

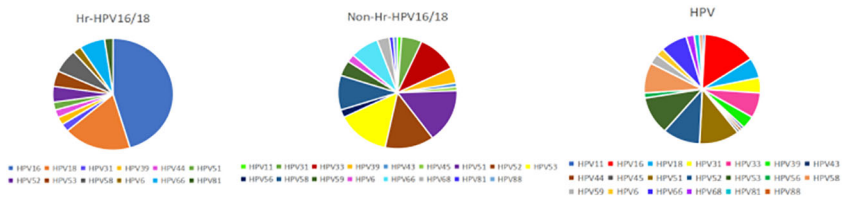

FIGURE 1
Prevalence of different HPV subtypes in infected groups. Hr-HPV16/18 (n = 28): HPV16/18 high-risk infection group; non-Hr-HPV16/18 (n = 62): non-HPV16/18 high-risk infection group; HPV: HPV-infected group (n = 90).

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TABLE 1 Descriptive characteristics of all samples included in the study.

Variable	Subcategory	Controls (n = 45)	Hr-HPV16/18 (n = 28)	Non-Hr-HPV16/18 (n = 62)	*p*
Age, yrs		36 (IQR = 17)	37 (IQR = 18)	41.5 (IQR = 10.75)	0.134
SIL grade	Low grade	2 (4.44%)	1 (3.57%)	9 (14.52%)	0.119
	High grade	1 (2.22%)	4 (14.29%)	6 (9.68%)	
	ASC-US	2 (4.44%)	10 (35.71%)	23 (37.10%)	
	ASC-H	1 (2.22%)	4 (14.29%)	0 (0.00%)	
Menopause	YES	6 (13.33%)	2 (7.14%)	11 (17.74%)	0.405
	NO	39 (86.67%)	26 (92.86%)	51 (82.26%)	
Non-menstrual bleeding	YES	30 (66.67%)	9 (32.14%)	20 (32.26%)	<0.001
	NO	15 (33.33%)	19 (67.86%)	42 (67.74%)	
IUD/Tubal ligation/condom	YES	20 (44.44%)	10 (35.71%)	24 (38.71%)	0.318
	NO	25 (55.56%)	18 (64.29%)	38 (61.29%)	
Trichomonad	YES	1 (2.22%)	0 (0.00%)	0 (0.00%)	
	NO	44 (97.78%)	28 (100.00%)	62 (100.00%)	
No. of pregnancies		4 (IQR = 2)	3 (IQR = 2)	3 (IQR = 2)	0.596
No. of births		2 (IQR = 1.5)	2 (IQR = 1)	2 (IQR = 1)	0.913
No. of miscarriages		1 (IQR = 2)	0 (IQR = 1)	0 (IQR = 2)	0.786
No. of white blood cells		6.11 (IQR = 4.36)	7.34 (IQR = 1.75)	5.60 (IQR = 2.62)	0.076
Cleaning degree of vagina	I	4 (8.89%)	4 (14.29%)	9 (14.52%)	0.101
	II	25 (55.56%)	22 (78.57%)	40 (64.52%)	
	III	6 (13.33%)	1 (3.57%)	3 (4.84%)	
	IV	3 (6.67%)	0 (0.00%)	2 (3.23%)	
UU (Ureaplasma urealyticum)	+	1 (2.22%)	3 (10.71%)	6 (9.68%)	
	–	0 (0.00%)	1 (3.57%)	3 (4.84%)	
MH (M. hominis)	+	1 (2.22%)	2 (7.14%)	4 (6.45%)	
	–	0 (0.00%)	2 (7.14%)	5 (8.06%)	
CT (Chlamydia trachomatis)	+	0 (0.00%)	1 (3.57%)	2 (3.23%)	
	–	1 (4.44%)	3 (10.71%)	7 (11.29%)	
Cervical intraepithelial neoplasm		3 (6.67%)	2 (7.14%)	6 (9.68%)	

Hr-HPV16/18, HPV16/18 high-risk infection group; Non-Hr-HPV16/18, non-HPV16/18 high-risk infection group; SIL, squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells: cannot exclude high-grade squamous intraepithelial lesion; IUD, intrauterine device.

Prevotella bivia (*P. bivia*) (0.23% vs.0.64% vs.1.79%, $p < 0.05$) (Figure 6).

Features of vaginal microflora in individuals infected with single, dual, or multiple HPVs

We divided patients into three groups: infected by a single HPV type (n = 56), infected by two HPV types (n = 26), and infected by multiple HPV types (n = 8). We further analyzed whether vaginal microbial diversity would be influenced by the number of infected HPV types. Women infected with multiple HPV types tended to have higher alpha diversity than those infected with a single HPV type, but the difference was not statistically significant ($p > 0.05$) (Figures 7, 8). To identify bacteria specifically linked with HPV infection status, LDA with effect size (LEfSe) modeling was conducted (Figure 9). The larger LDA indicated the greater difference of the species. In the single HPV type group, the predominant species was *Klebsiella* ($p <$

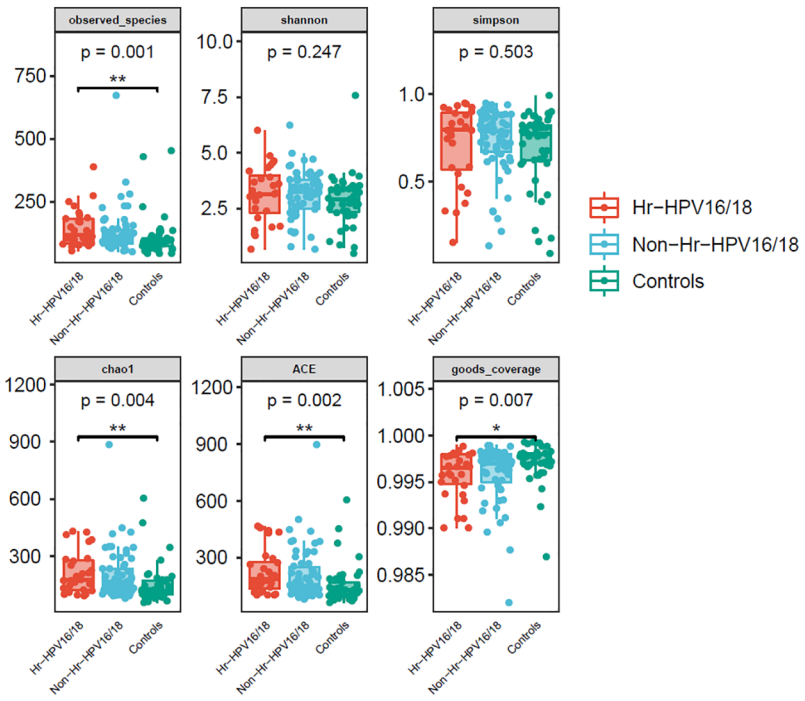

FIGURE 2
Comparison of vaginal microbial alpha diversity index (observed - species, Shannon, Simpson, Chao1, ACE, good - coverage) in infected and healthy individuals. The *p*-value on the top indicates the overall difference among three groups calculated using the Kruskal-Wallis nonparametric test method, and the asterisks on the top indicate a statistically significant difference between the two groups calculated using Dunn's test (*p < 0.05, * *p < 0.01).

0.05). In the dual HPV type group, the predominant species were *Parvimonas*, *unidentified Christensenellacea*, *Candidatus competibacter*, *unidentified Gammaproteobacteria*, *Terrimonas*, *Leisingera*, *Hyphomicrobium*, *Terrabacter*, *unidentified Alphaproteobacteria* and *Enhydrobacter* ($p < 0.05$). In the multiple HPV type group, the predominant species were *Ferruginibacter*, *Haloactinopolyspora*, *unidentified Rhizobiaceae*, *Blastochloris*, *Vibrio*, *Ornithinimicrobium*, *Tetragenococcus*, and *Castellaniella*.

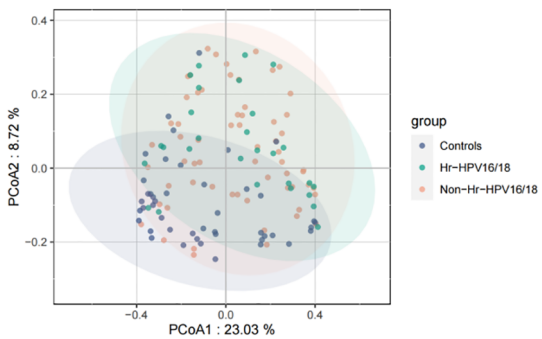

FIGURE 3
Principal coordinates analysis (PCoA) of variation in beta diversity of human vaginal bacterial communities in infected and healthy individuals based on unweighted UniFrac phylogenetic distance. Hr-HPV16/18 (n = 28): HPV16/18 high-risk infection group; Non-Hr-HPV16/18 (n = 62): non-HPV16/18 high-risk infection group; controls (n = 45): non-HPV infection group.

Vaginal flora features in patients with HPV clearance

We further analyzed whether vaginal flora affects HPV clearance. There were 26 patients who had HPV clearance within a year, with most turning negative within six months. Analysis of vaginal flora characteristics showed the women with HPV clearance had significantly lower bacterial diversity, with scores of 0.046 for Chao1, and 0.04 for ACE diversity, respectively (Figure 10).

The top 20 bacteria were analyzed by heat mapping (Figure 11). At the phylum level, no significant changes were indicated between patients with HPV clearance and HPV persistence, for Firmicutes (53.53% vs. 57.55%, $p > 0.05$), Proteobacteria (21.90% vs. 15.24%, $p > 0.05$), Actinobacteria (13.59% vs. 12.09%, $p > 0.05$), Fusobacteria (4.76% vs. 8.33%, $p > 0.05$), and Bacteroidetes (5.40% vs. 5.51%, $p > 0.05$). At the genus

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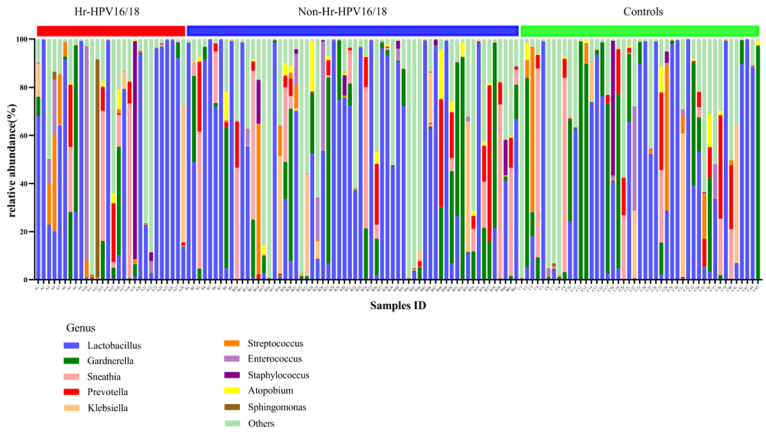

FIGURE 4
Vaginal flora at the genus level in infected and healthy individuals.

level, no significant changes were observed for *Lactobacillus* (45.79% vs. 43.83%, $p > 0.05$), *Gardnerella* (12.67% vs. 10.03%, $p > 0.05$), *Sneathia* (4.75% vs. 8.11%, $p > 0.05$), *Prevotella* (4.55% vs. 3.87%, $p > 0.05$), *Klebsiella* (5.16% vs. 1.99%, $p > 0.05$), *Enterococcus* (3.22% vs. 2.12%, $p > 0.05$), *Streptococcus* (0.50% vs. 2.71%, $p > 0.05$), *Staphylococcus* (0.28%, vs. 2.19%, $p > 0.05$), *Atopobium* (0.77% vs. 1.40%, $p > 0.05$), and *Sphingomonas* (0.01% vs. 1.48%, $p > 0.05$) (Figure 12). At the species level, between patients who had cleared HPV vs. patients with persistent HPV, no changes were observed for *L. iners* (32.44% vs. 27.54%, $p > 0.05$), *S. amnii* (2.73% vs. 5.65%, $p > 0.05$), *E. faecalis* (2.87% vs. 1.61%, $p > 0.05$), *S haemolyticus* (0.26% vs. 1.62%, $p > 0.05$), *A. vaginae* (0.77% vs.1.40%, $p > 0.05$), *Streptococcus intermedius* (*S. intermedius*) (0.31% vs. 1.33%, $p > 0.05$), *S. sanguinegens* (0.84% vs. 1.11%, $p > 0.05$), *Prevotella timonensis* (*P. timonensis*) (1.61% vs. 0.70%, $p > 0.05$), *P. amnii* (0.13% vs. 1.27%, $p > 0.05$), and *L. gasseri* (0.67% vs. 1.05%, $p > 0.05$).

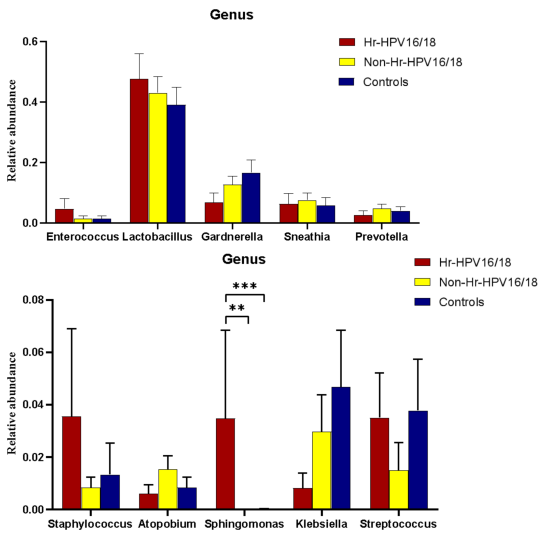

FIGURE 5
Comparison of relative abundance of the top 10 vaginal microflora genera between the Hr-HPV16/18 (n = 28), non-Hr-HPV16/18 (n = 62), and healthy (n = 45) individuals. *p* - values of 0.05 indicate a statistically significant difference, **p < 0.01, ***p < 0.001.

To identify bacteria specifically linked with HPV clearance, LEfSe modeling was conducted (Figure 13). Our results showed that patients who had developed a persistent HPV infection had significantly higher levels of Erysipelotrichia (class level), Bacteroidaceae, Erysipelotrichaceae, Helicobacteraceae, Neisseriaceae, Streptococcaceae (family level), Erysipelotrichales, Flavobacteriales (order level), and *Fusobacterium*, *Bacteroides*, *Neisseria*, and *Helicobacter* (genus level) than patients who had cleared HPV ($p < 0.05$).

Factors associated with HPV infection

Spearman correlation analysis was exploited to analyze the correlation between HPV infection and clinical biomarkers. Our results showed that vaginal cleanliness and non-menstrual bleeding were two related factors, with correlation coefficients of $\rho = 0.195$ ($p < 0.05$) and $\rho = 0.327$ ($p < 0.05$), respectively. The main HPV subtypes and the dominant bacteria detected in all patient samples were selected for a chord diagram (Figure 14). Each HPV subtype was linked to a dominant bacterium. The wider the link was, the larger the number of dominant bacteria in patients of this subtype. *L. iners* was the bacterial species most connected with HPV subtypes. The second was the *S. amnii*, and the third was the *Prevotella*. In

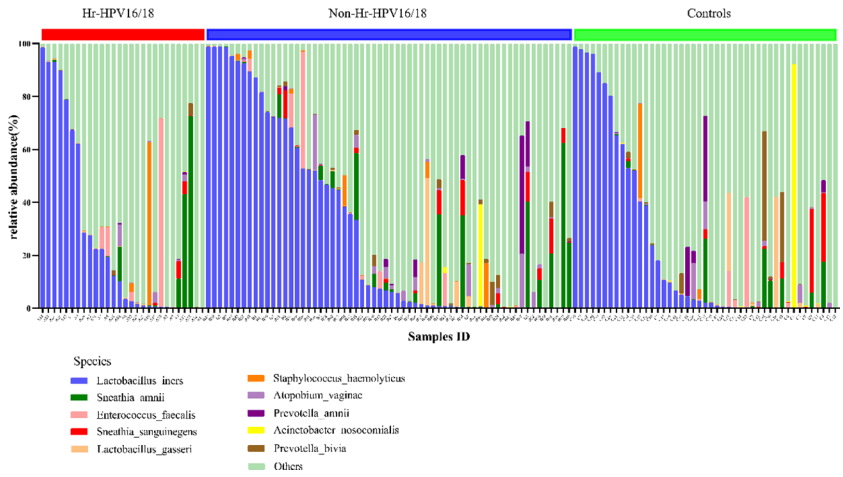

FIGURE 6
Vaginal flora at the species level of Hr-HPV16/18 (n = 28), non-Hr-HPV16/18 (n = 62), and controls (n = 45).

addition, we found that the dominant bacteria with the highest prevalence in HPV-positive samples were *L. iners* – dominant (n = 49, 54.44%) and *S. amnii*-dominant (n = 10, 11.11%). The top 3 dominant bacteria in the HPV-persistent group were *L. iners* (n = 34, 53.13%), *S. amnii* (n = 9, 14.06%), and *L. delbrueckii* (n = 3, 4.69%).

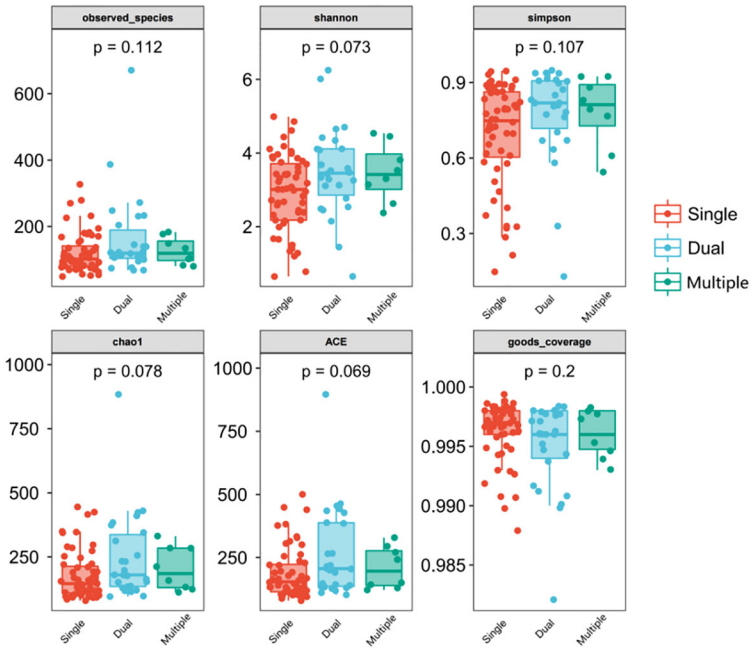

FIGURE 7
Comparison between alpha diversity index (observed - species, Shannon, Simpson, Chao1, ACE, good-coverage) of infected and healthy individuals. The *p*-value on the top indicates the overall difference among three groups calculated using the Kruskal-Wallis nonparametric test method, and the asterisks on the top indicate a statistically significant difference between the two groups calculated using Dunn's test (* $p < 0.05$, ** $p < 0.01$). Single (n = 56): infected with a single HPV subtype; dual (n = 26): infected with two HPV subtypes; Multiple (n = 8): infected with three or more HPV subtypes.

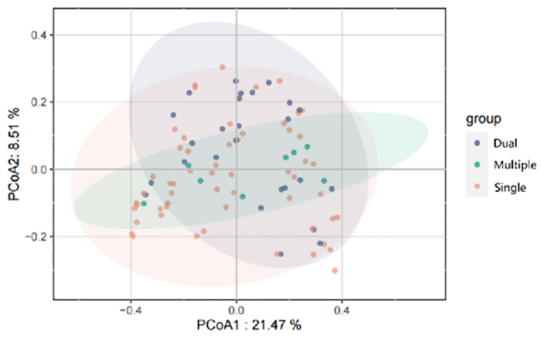

FIGURE 8
Principal-coordinates analysis (PCoA) of variation in beta diversity of human vaginal bacterial communities in infected and healthy individuals, based on unweighted UniFrac phylogenetic distance.

Discussion

The vaginal flora is an important factor in modulating the vaginal mucosa microenvironment against viral infections (Nieves-Ramírez et al., 2021). Viral infection may also disturb the normal structure and composition of the vaginal flora. In this study, we found that bacterial components vary between the HPV infectious subgroups and healthy controls. The dominant bacteria with the highest prevalence in HPV-positive group were *L. iners*, *S. amnii*, and *Prevotella*. Particularly, *S. amnii* was significantly higher, *L. gasseri, P. bivia*, and *A. vaginae* were significantly lower in the Hr-HPV 16/18 group than those in healthy individuals. This decrease in the predominating protective bacteria leads to the increase of vaginal pH levels, the weakening of pathogenic defense ability and the damage of mucosal barriers (Gillet et al., 2012; Łaniewski et al., 2018). The differences in the composition of vaginal flora may be the basis for dysbiotic patterns associated with HPV infection and cervical cancer in different female populations (Bychkovsky et al., 2016; Curty et al., 2017; Romero-Morelos et al., 2019; Nieves-Ramírez et al., 2021).

The vaginal flora is a complicated ecosystem affected by a variety of factors, including environment, host, and ethnicity (Serrano et al., 2019; Moosa et al., 2020). It has also been reported that different ethnic groups have different characteristics of the cervicovaginal microbiota (Casey et al., 2012). The prevalence of *Lactobacillus* spp. as the dominant microbiota is higher in Caucasian and Asian women compared to Hispanic and Black women (Ravel et al., 2011; Anahtar et al., 2015). These differences may be resulted from genetic factors affecting mucosal immunity or metabolic pathways, leading to preferred conditions for specific species, or they may the consequence of differences in different hygiene practices (Mitra et al., 2016). In this study, all participants are from Chinese, which allows us to limit confounders due to race-ethnic diversity that may affect the vaginal flora (Serrano et al., 2019). Covariates that may impact on the vaginal flora include smoking status, time within the menstrual cycle, sexual behavior, use of hormonal contraceptives or copper intrauterine devices, as well as ethnic background (Cherpes et al., 2008; Srinivasan et al., 2010; Gajer et al., 2012; Romero et al., 2014). Our results show that vaginal flora is dominated by *Lactobacillus*, followed by

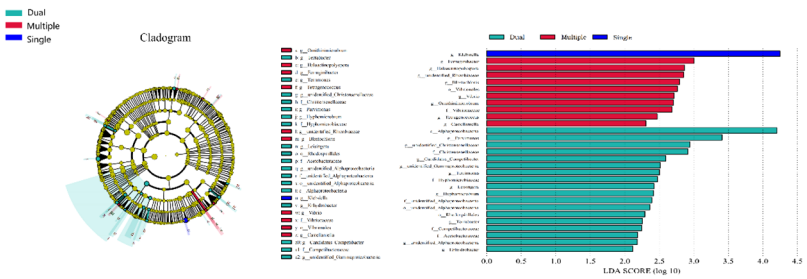

FIGURE 9
LEfSe analysis comparing microbial variations at the genus level in patients infected with one, two, or multiple HPV subtypes. LEfSe cladogram representing differentially abundant taxa ($p < 0.05$). LDA scores as calculated by LEfSe of taxa are differentially abundant among groups. Only taxa with LDA scores of >2 were presented.

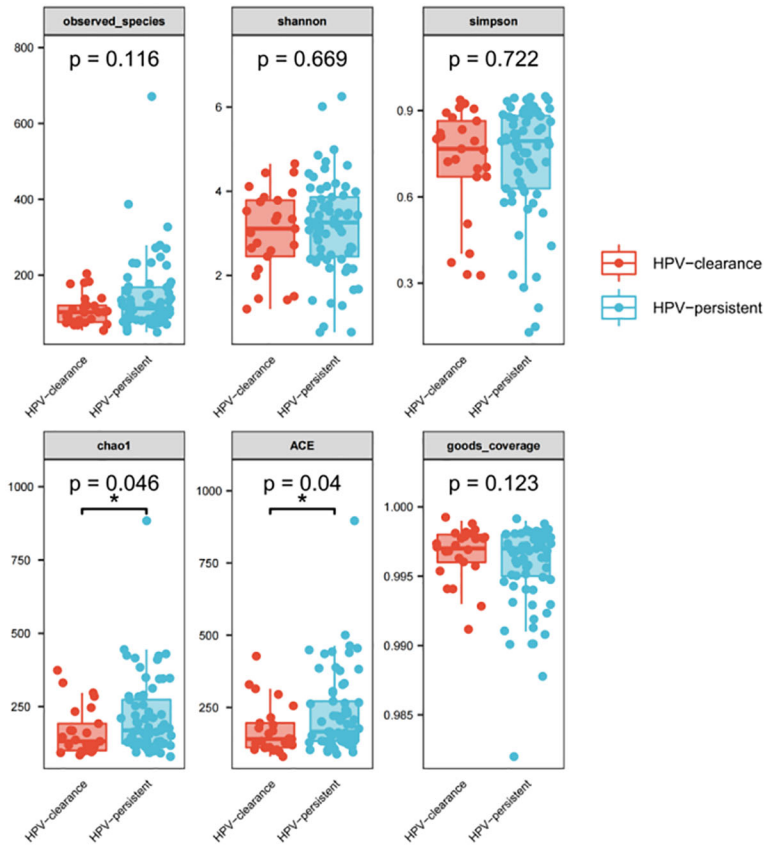

FIGURE 10
Alpha diversity index (observed - species, Shannon, Simpson, Chao1, ACE, good-coverage) in the HPV-cleared and HPV-persistent groups. The *p*-value on the top indicates the overall difference among three groups calculated using the Kruskal-Wallis nonparametric test method, and the asterisks on the top indicate a statistically significant difference between the two groups calculated using Dunn's test (* $p < 0.05$, ** $p < 0.01$).

Gardnerella, Sneathia, Prevotella, Klebsiella, Streptococcus, Enterococcus, and Staphylococcus, either in HPV-infected or in healthy individuals. Several vaginal microbes, such as increased *Gardnerella, Fusobacteria, Bacillus cohnii, Dialister, Prevotella*, and *Mycoplasma*, are associated with dysbiosis that would lead to instability in the microenvironment, which in turn may allow key risk factors to have an impact on cervical cancer (Gao et al., 2013; Ritu et al., 2019; Usyk et al., 2020; Kovachev, 2020).. In our study, the bacterial genera *Sphingomonas* showed a significant difference between the Hr-HPV16/18 group and the healthy controls. In the top 10 bacterial species, the abundance of *S. amnii*, *E. faecalis*, and *S. haemolyticus* were two-fold higher, and *P. amnii*, *A. nosocomialis*, *S. sanguinegens*, *L. gasseri* were two-fold lower in the Hr-HPV 16/18 group than in the controls. *L. gasseri*, rather than *L. iners*, was significantly different among the three groups. *Sneathia* spp. has frequently been associated with HPV positivity (Lee et al., 2013), but its different species varied in HPV-infected subgroups. Therefore, the pattern of flora associated with HPV infection may be unique in different populations. Identifying changes in bacterial composition of HPV-associated cervical cancers may offer new ideas into potential target populations and potential biomarkers of disease and disease-state (Lin et al., 2020).

The local cervical microenvironment can also affect the natural course of HPV infection (Castle and Giuliano, 2003). The previous study revealed that transient and persistent HPV16 infections, comparing with no HPV infection, are related to vaginal flora dominated by non-*Lactobacillus* species (Berggrund et al., 2020). We then studied whether specific vaginal bacteria are associated with HPV clearance. Our results show that bacterial genera (*Sneathia, Streptococcus, Staphylococcus* and *Sphingomonas*), and species (*S. amnii, S. haemolyticus, S. intermedius*, and *P. amnii*), were observed significantly higher in the persistent HPV group than those in the cleared HPV group. LDA analysis showed that *Fusobacterium*, *Bacteroides*, *Neisseria*, and *Helicobacter* are characteristic bacterial genera that are significantly different between patients with persistent HPV and in patients who cleared HPV. Women with a certain specific composition of vaginal flora might be more susceptible to HPV infection or

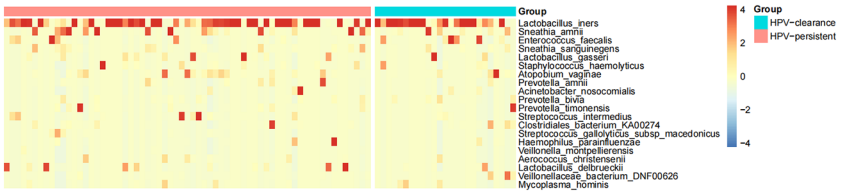

FIGURE 11
Heat map analysis of bacterial species found in the vaginal flora of 90 women. Each vertical line represents one sample. Different colors indicate relative abundance: red represents a high proportion and blue represents a low proportion. HPV-clearance: patients who cleared HPV; HPV-persistent: patients who did not clear HPV.

show faster progression of dysplasia (Norenhag et al., 2020). Distinguishing bacterial features associated with HPV clearance in patients will be helpful for early intervention and reversal of persistent infection, which will contribute to reducing the incidence of cervical cancer.

Vaginal *Lactobacilli* can exert vaginal protection through multiple mechanisms. For example, *Lactobacilli* can offer broad-spectrum protection by producing lactic acid, bacteriocins, and biosurfactants, and forming barriers against pathogenic infections in the vaginal microenvironment by adhering to the mucosa (Mitra et al., 2016; Piyathilake et al., 2016; Łaniewski et al., 2019; Ilhan et al., 2019; Borgogna et al., 2020). For treatment options, the addition of exogenous probiotics, i.e., *Lactobacillus* can improve the treatment of cervicovaginal dysbiosis and persistent HPV infections (Qingqing et al., 2021). In comparision with short-term use, long-term use of vaginal probiotics containing *Lactobacillus* spp. is related with increased clearance of HPV (Palma et al., 2018). However, the therapeutic effect varies greatly. Among the main components of a healthy vaginal flora is the presence of *Lactobacillus* spp., which includes *L. crispatus, L. iners, L. jensenii*, and *L. gasseri* (Łaniewski et al., 2018; Kovachev, 2020). A previous study suggested that bacterial community state types dominated by *L. gasseri* might be related with the fastest clearance of acute HPV infection (Brotman et al., 2014). Unlike *L. crispatus, L. iners* produces small amounts of lactic acid without the production of reported host-protective peptide. Cervicovaginal microbiota of transiently HPV-infected women is dominated by *L. iners* (Qingqing et al., 2021), probably because *L. iners* are able to adapt to various pH environments and apparently lack genes for the synthesis of bacteriocin, all of which creates conditions for abnormal cervicovaginal bacteria to proliferate (Macklaim et al., 2011; Mitra et al., 2016). The predominant microbiota in vaginal flora samples was *L. crispatus* or *L. iners*, whereas individuals with a low-*Lactobacillus* vaginal microbiota usually have the colonizedzation of bacteria such as *Gardnerella, Prevotella*, and *Sneathia* (Ravel et al., 2011; Callahan et al., 2017; Serrano et al., 2019). In our study, only 28.89% of

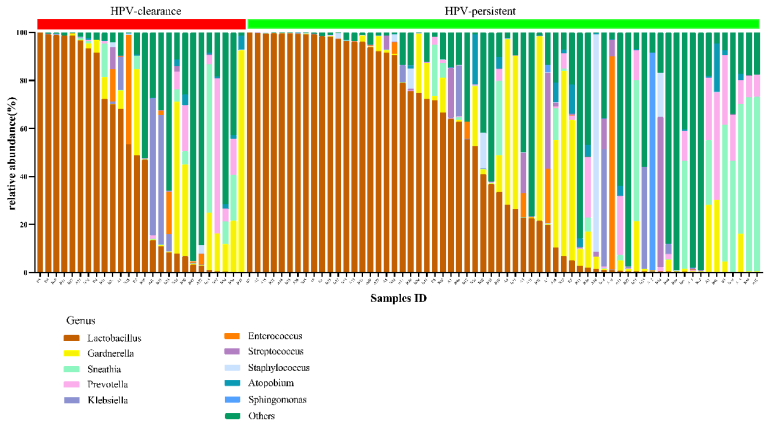

FIGURE 12
Vaginal flora at the genus level of HPV-cleared (n = 26) and HPV-persistent (n = 64) patients.

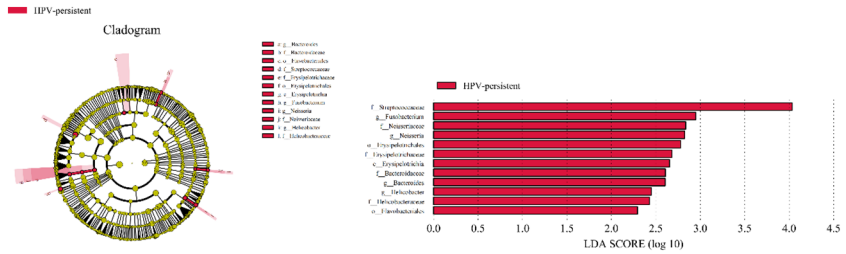

FIGURE 13
LEfSe analysis comparing microbial variations at the genus level in infected and healthy individuals. LEfSe cladogram representing differentially abundant taxa ($p < 0.05$). LDA scores as calculated by LEfSe of taxa are differentially abundant among groups. Only taxa with LDA scores of >2 are presented.

patients cleared HPV following treatment with IFN plus vaginal *Lactobacillus* spp. Higher levels of non- *Lactobacillus* dominant bacteria, including *S. amnii, E. faecalis, S. haemolyticus, S. intermedius*, *P. amnii*, and *P. timonensis*, were found in the HPV-persistent group than those in the HPV-cleared group, indicating that women with a high abundance of these bacteria have more difficulty in clearing HPV (Ritu et al., 2019; Chao et al., 2020).

We further classified patients based on their HPV subtypes and assessed the relationship between HPV phylogenetic groups and the composition of the vaginal flora. Our results indicate that *L. iners* is the primary bacterial species that is connected with HPV subtypes. Some studies have demonstrated a link between greater cervical microbiome (CVM) diversity and prevalence of Hr-HPV infection and/or cervical abnormalities (vs. HPV negative) (Audirac-Chalifour et al., 2016; Dareng et al., 2016). Increased alpha diversity was associated with Hr-HPV positivity that was associated with increasing disease severity (Mitra et al., 2015; Klein et al., 2019). Consistent with the previous study, women with multiple HPV types infection showed higher bacterial diversity, with higher diversity being displayed by women with a single HPV type infection than women with no HPV infection (Cheng et al., 2020). Of note, in the HPV-persistent group, women showed significantly higher bacterial diversity than the HPV-cleared group. Presently, evidence on the relationship between CVM diversity and cervical neoplasia severity is conflicting (Mitra et al., 2015; Seo et al., 2016). Because most studies focusing on the natural course of HPV and the microbiome are cross-sectional, it is difficult to decipher potential causality. The potential mechanisms underlying the potential interactions between HPV and microbiota need to be revealed (Cheng et al., 2020).

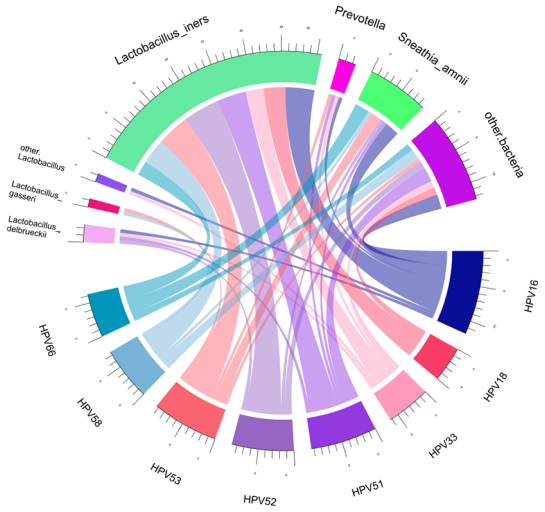

FIGURE 14
Chord diagram shows the relationship between HPV subtypes and dominant vaginal bacteria. The width of the strings (connecting lines) in the chord diagram shows the extent and proportion of the association between different HPV subtypes and dominant vaginal bacteria. The wider the width of the connecting lines, the higher the proportion. Different colors distinguish between different relationships.

Probiotics are believed to exert a helpful influence on a wide range of diseases. Compared with the development of novel anti-inflammatory drugs, it may be less costly to find novel approaches, i.e., probiotics that can change the vaginal flora environment by playing a direct role in vaginal flora (Nami et al., 2014; Nami et al., 2014; Eslami et al., 2016; Wang et al., 2018). Currently, probiotic strains of *lactobacillus* administered vaginally by suppository or vaginal ovule have been explored (Knackstedt et al., 2020). Our clinical experience shows that probiotics, as adjunctive therapy for interferon, have a good therapeutic effect on HPV clearance, but also have some efficacy in the clearance of chlamydia and mycoplasma (data not shown). Thus, it is crucial to provide a completely new method to cure diseases by monitoring specific bacterium associated with HPV infection and controlling vaginal flora (Li et al., 2020).

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA913296

Ethics statement

The studies involving human participants were reviewed and approved by The Research Ethics Boards at the First Affiliated Hospital of Shantou University Medical College (No. 201561). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JC conceived the study and designed the experiments. MZ and XL performed experiments and analyzed data. XJ and JC wrote the manuscript. FY, SX, XH, and XC revised the manuscript extensively. All of the authors have discussed and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1036869/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Schematic diagram of the experimental design from the sample collection to statistic alanalyses.

References

Anahtar, M. N., Byrne, E. H., Doherty, K. E., Bowman, B. A., Yamamoto, H. S., Soumillon, M., et al. (2015). Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity.* 42 (5), 965–976. doi: 10.1016/j.immuni.2015.04.019

Audirac-Chalifour, A., Torres-Poveda, K., Bahena-Román, M., Téllez-Sosa, J., Martínez-Barnetche, J., Cortina-Ceballos, B., et al. (2016). Cervical microbiome and cytokine profile at various stages of cervical cancer: A pilot study. *PLos One* 11 (4), e0153274. doi: 10.1371/journal.pone.0153274

Berggrund, M., Gustavsson, I., Aarnio, R., Lindberg, J. H., Sanner, K., Wikström, I., et al. (2020). Temporal changes in the vaginal microbiota in self-samples and its association with persistent HPV16 infection and CIN2. *Virol. J.* 17 (1), 147. doi: 10.1186/s12985-020-01420-z

Borgdorff, H., Gautam, R., Armstrong, S. D., Xia, D., Ndayisaba, G. F., van Teijlingen, N. H., et al. (2016). Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier. *Mucosal Immunol.* 9 (3), 621–633. doi: 10.1038/mi.2015.86

Borgogna, J. C., Shardell, M. D., Santori, E. K., Nelson, T. M., Rath, J. M., Glover, E. D., et al. (2020). The vaginal metabolome and microbiota of cervical HPV-positive and HPV-negative women: a cross-sectional analysis. *BJOG.* 127 (2), 182–192. doi: 10.1111/1471-0528.15981

Breshears, L. M., Edwards, V. L., Ravel, J., and Peterson, M. L. (2015). Lactobacillus crispatus inhibits growth of gardnerella vaginalis and neisseria gonorrhoeae on a porcine vaginal mucosa model. *BMC Microbiol.* 15, 276. doi: 10.1186/s12866-015-0608-0

Brotman, R. M., Shardell, M. D., Gajer, P., Tracy, J. K., Zenilman, J. M., Ravel, J., et al. (2014). Interplay between the temporal dynamics of the vaginal microbiota and human papillomavirus detection. *J. Infect. dis* 210 (11), 1723–1733. doi: 10.1093/infdis/jiu330

Brusselaers, N., Shrestha, S., van de Wijgert, J., and Verstraelen, H. (2019). Vaginal dysbiosis and the risk of human papillomavirus and cervical cancer: systematic review and meta-analysis. *Am. J. obstetrics gynecol* 221 (1), 9–18.e8. doi: 10.1016/j.ajog.2018.12.011

Bychkovsky, B. L., Ferreyra, M. E., Strasser-Weippl, K., Herold, C. I., de Lima Lopes, G.Jr., Dizon, D. S., et al. (2016). Cervical cancer control in Latin America: A call to action. *Cancer.* 122 (4), 502–514. doi: 10.1002/cncr.29813

Bzhalava, D., Mühr, L. S., Lagheden, C., Ekström, J., Forslund, O., Dillner, J., et al. (2014). Deep sequencing extends the diversity of human papillomaviruses in human skin. *Sci. Rep.* 4, 5807. doi: 10.1038/srep05807

Callahan, B. J., DiGiulio, D. B., Goltsman, D. S. A., Sun, C. L., Costello, E. K., Jeganathan, P., et al. (2017). Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc. Natl. Acad. Sci. United States America* 114 (37), 9966–9971. doi: 10.1073/pnas.1705899114

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7 (5), 335–336. doi: 10.1038/nmeth.f.303

Casey, B. J., Somerville, L. H., Gotlib, I. H., Ayduk, O., Franklin, N. T., Askrend, M. K., et al. (2012). Behavioral and neural correlates of delay of gratification 40 years later. *Proc. Natl. Acad. Sci. U.S.A. 2011 Vol 108 No. 36:14998-5003 Ann. neurosciences* 19 (1), 27–28. doi: 10.5214/ans.0972.7531.180407

Castle, P. E., and Giuliano, A. R. (2003). Chapter 4: Genital tract infections, cervical inflammation, and antioxidant nutrients–assessing their roles as human

papillomavirus cofactors. *J. Natl. Cancer Inst Monogr.* (31), 29–34. doi: 10.1093/oxfordjournals.jncimonographs.a003478

Chan, C. K., Aimagambetova, G., Ukybassova, T., Kongrtay, K., and Azizan, A. (2019). Human papillomavirus infection and cervical cancer: Epidemiology, screening, and vaccination-review of current perspectives. *J. Oncol.* 2019, 3257939. doi: 10.1155/2019/3257939

Chang, A. H., and Parsonnet, J. (2010). Role of bacteria in oncogenesis. *Clin. Microbiol. Rev.* 23 (4), 837–857. doi: 10.1128/CMR.00012-10

Chao, X., Sun, T., Wang, S., Tan, X., Fan, Q., Shi, H., et al. (2020). Research of the potential biomarkers in vaginal microbiome for persistent high-risk human papillomavirus infection. *Ann. Trans. Med.* 8 (4), 100. doi: 10.21037/atm.2019.12.115

Cheng, L., Norenhag, J., Hu, Y. O. O., Brusselaers, N., Fransson, E., Ährlund-Richter, A., et al. (2020). Vaginal microbiota and human papillomavirus infection among young Swedish women. *NPJ biofilms microbiomes* 6 (1), 39. doi: 10.1038/s41522-020-00146-8

Cherpes, T. L., Hillier, S. L., Meyn, L. A., Busch, J. L., and Krohn, M. A. (2008). A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. *Sexually transmitted dis* 35 (1), 78–83. doi: 10.1097/OLQ.0b013e318156a5d0

Clifford, G. M., Smith, J. S., Plummer, M., Muñoz, N., and Franceschi, S. (2003). Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br. J. cancer* 88 (1), 63–73. doi: 10.1038/sj.bjc.6600688

Curty, G., Costa, R. L., Siqueira, J. D., Meyrelles, A. I., Machado, E. S., Soares, E. A., et al. (2017). Analysis of the cervical microbiome and potential biomarkers from postpartum HIV-positive women displaying cervical intraepithelial lesions. *Sci. Rep.* 7 (1), 17364. doi: 10.1038/s41598-017-17351-9

Dareng, E. O., Ma, B., Famooto, A. O., Adebamowo, S. N., Offiong, R. A., Olaniyan, O., et al. (2016). Prevalent high-risk HPV infection and vaginal microbiota in Nigerian women. *Epidemiol. infection* 144 (1), 123–137. doi: 10.1017/S0950268815000965

DiGiulio, D. B., Callahan, B. J., McMurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., et al. (2015). Temporal and spatial variation of the human microbiota during pregnancy. *Proc. Natl. Acad. Sci. United States America* 112 (35), 11060–11065. doi: 10.1073/pnas.1502875112

Di Paola, M., Sani, C., Clemente, A. M., Iossa, A., Perissi, E., Castronovo, G., et al. (2017). Characterization of cervico-vaginal microbiota in women developing persistent high-risk human papillomavirus infection. *Sci. Rep.* 7 (1), 10200.

Du, J., Näsman, A., Carlson, J. W., Ramqvist, T., and Dalianis, T. (2011). Prevalence of human papillomavirus (HPV) types in cervical cancer 2003-2008 in Stockholm, Sweden, before public HPV vaccination. *Acta Oncol. (Stockholm Sweden)* 50 (8), 1215–1219. doi: 10.3109/0284186X.2011.584556

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10 (10), 996–998. doi: 10.1038/nmeth.2604

Eslami, S., Hadjati, J., Motevaseli, E., Mirzaei, R., Farashi Bonab, S., Ansaripour, B., et al. (2016). Lactobacillus crispatus strain SJ-3C-US induces human dendritic cells (DCs) maturation and confers an anti-inflammatory phenotype to DCs. *APMIS Acta pathologica microbiologica immunologica Scandinavica* 124 (8), 697–710. doi: 10.1111/apm.12556

Fernandes, J. V., DEMF, T. A., DEA, J. C., Cobucci RN, M. G., VS, A., DEA, J. M., et al. (2015). Link between chronic inflammation and human papillomavirus-induced carcinogenesis (Review). *Oncol. letters* 9 (3), 1015–1026. doi: 10.3892/ol.2015.2884

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schütte, U. M., Zhong, X., et al. (2012). Temporal dynamics of the human vaginal microbiota. *Sci. Trans. Med.* 4 (132), 132ra52. doi: 10.1126/scitranslmed.3003605

Gao, W., Weng, J., Gao, Y., and Chen, X. (2013). Comparison of the vaginal microbiota diversity of women with and without human papillomavirus infection: a cross-sectional study. *BMC Infect. dis* 13, 271. doi: 10.1186/1471-2334-13-271

Gillet, E., Meys, J. F., Verstraelen, H., Verhelst, R., De Sutter, P., Temmerman, M., et al. (2012). Association between bacterial vaginosis and cervical intraepithelial neoplasia: systematic review and meta-analysis. *PLos One* 7 (10), e45201. doi: 10.1371/journal.pone.0045201

Godoy-Vitorino, F., Romaguera, J., Zhao, C., Vargas-Robles, D., Ortiz-Morales, G., Vázquez-Sánchez, F., et al. (2018). Cervicovaginal fungi and bacteria associated with cervical intraepithelial neoplasia and high-risk human papillomavirus infections in a Hispanic population. *Front. Microbiol.* 9, 2533. doi: 10.3389/fmicb.2018.02533

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., et al. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* 21 (3), 494–504. doi: 10.1101/gr.112730.110

Ilhan, Z. E., Łaniewski, P., Thomas, N., Roe, D. J., Chase, D. M., and Herbst-Kralovetz, M. M. (2019). Deciphering the complex interplay between microbiota, HPV, inflammation and cancer through cervicovaginal metabolic profiling. *EBioMedicine.* 44, 675–690. doi: 10.1016/j.ebiom.2019.04.028

Kabuki, T., Saito, T., Kawai, Y., Uemura, J., and Itoh, T. (1997). Production, purification and characterization of reutericin 6, a bacteriocin with lytic activity produced by lactobacillus reuteri LA6. *Int. J. Food Microbiol.* 34 (2), 145–156. doi: 10.1016/S0168-1605(96)01180-4

Klein, C., Gonzalez, D., Samwel, K., Kahesa, C., Mwaiselage, J., Aluthge, N., et al. (2019). Relationship between the cervical microbiome, HIV status, and precancerous lesions. *mBio* 10 (1), e02785-18. doi: 10.1128/mBio.02785-18

Knackstedt, R., Knackstedt, T., and Gatherwright, J. (2020). The role of topical probiotics in skin conditions: A systematic review of animal and human studies and implications for future therapies. *Exp. Dermatol.* 29 (1), 15–21. doi: 10.1111/exd.14032

Kovachev, S. M. (2020). Cervical cancer and vaginal microbiota changes. *Arch. Microbiol.* 202 (2), 323–327. doi: 10.1007/s00203-019-01747-4

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31 (9), 814–821. doi: 10.1038/nbt.2676

Łaniewski, P., Barnes, D., Goulder, A., Cui, H., Roe, D. J., Chase, D. M., et al. (2018). Linking cervicovaginal immune signatures, HPV and microbiota composition in cervical carcinogenesis in non-Hispanic and Hispanic women. *Sci. Rep.* 8 (1), 7593. doi: 10.1038/s41598-018-25879-7

Łaniewski, P., Cui, H., Roe, D. J., Barnes, D., Goulder, A., Monk, B. J., et al. (2019). Features of the cervicovaginal microenvironment drive cancer biomarker signatures in patients across cervical carcinogenesis. *Sci. Rep.* 9 (1), 7333. doi: 10.1038/s41598-019-43849-5

Lee, J. E., Lee, S., Lee, H., Song, Y. M., Lee, K., Han, M. J., et al. (2013). Association of the vaginal microbiota with human papillomavirus infection in a Korean twin cohort. *PLos One* 8 (5), e63514. doi: 10.1371/journal.pone.0063514

Lehtinen, M., Baussano, I., Paavonen, J., Vänskä, S., and Dillner, J. (2019). Eradication of human papillomavirus and elimination of HPV-related diseases - scientific basis for global public health policies. *Expert Rev. Vaccines* 18 (2), 153–160. doi: 10.1080/14760584.2019.1568876

Lin, D., Kouzy, R., Abi Jaoude, J., Noticewala, S. S., Delgado Medrano, A. Y., Klopp, A. H., et al. (2020). Microbiome factors in HPV-driven carcinogenesis and cancers. *PLos pathogens* 16 (6), e1008524. doi: 10.1371/journal.ppat.1008524

Li, Y., Yu, T., Yan, H., Li, D., Yu, T., Yuan, T., et al. (2020). Vaginal microbiota and HPV infection: Novel mechanistic insights and therapeutic strategies. *Infection Drug resistance* 13, 1213–1220. doi: 10.2147/IDR.S210615

Macklaim, J. M., Clemente, J. C., Knight, R., Gloor, G. B., and Reid, G. (2015). Changes in vaginal microbiota following antimicrobial and probiotic therapy. *Microbial Ecol. Health disease* 26, 27799. doi: 10.3402/mehd.v26.27799

Macklaim, J. M., Gloor, G. B., Anukam, K. C., Cribby, S., and Reid, G. (2011). At The crossroads of vaginal health and disease, the genome sequence of lactobacillus iners AB-1. *Proc. Natl. Acad. Sci. United States America* 108 Suppl 1 (Suppl 1), 4688–4695. doi: 10.1073/pnas.1000086107

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–12. doi: 10.14806/ej.17.1.200

Mastromarino, P., Di Pietro, M., Schiavoni, G., Nardis, C., Gentile, M., and Sessa, R. (2014). Effects of vaginal lactobacilli in chlamydia trachomatis infection. *Int. J. Med. Microbiol. IJMM.* 304 (5-6), 654–661. doi: 10.1016/j.ijmm.2014.04.006

Mitra, A., MacIntyre, D. A., Lee, Y. S., Smith, A., Marchesi, J. R., Lehne, B., et al. (2015). Cervical intraepithelial neoplasia disease progression is associated with increased vaginal microbiome diversity. *Sci. Rep.* 5, 16865. doi: 10.1038/srep16865

Mitra, A., MacIntyre, D. A., Marchesi, J. R., Lee, Y. S., Bennett, P. R., and Kyrgiou, M. (2016). The vaginal microbiota, human papillomavirus infection and cervical intraepithelial neoplasia: what do we know and where are we going next? *Microbiome* 4 (1), 58. doi: 10.1186/s40168-016-0203-0

Mitra, A., MacIntyre, D. A., Ntritsos, G., Smith, A., Tsilidis, K. K., Marchesi, J. R., et al. (2020). The vaginal microbiota associates with the regression of untreated cervical intraepithelial neoplasia 2 lesions. *Nat. Commun.* 11 (1), 1999. doi: 10.1038/s41467-020-15856-y

Moosa, Y., Kwon, D., de Oliveira, T., and Wong, E. B. (2020). Determinants of vaginal microbiota composition. *Front. Cell. infection Microbiol.* 10, 467. doi: 10.3389/fcimb.2020.00467

Nami, Y., Abdullah, N., Haghshenas, B., Radiah, D., Rosli, R., and Khosroushahi, A. Y. (2014). Assessment of probiotic potential and anticancer activity of newly isolated vaginal bacterium lactobacillus plantarum 5BL. *Microbiol. Immunol.* 58 (9), 492–502. doi: 10.1111/1348-0421.12175

Nami, Y., Abdullah, N., Haghshenas, B., Radiah, D., Rosli, R., and Yari Khosroushahi, A. (2014). A newly isolated probiotic enterococcus faecalis strain from vagina microbiota enhances apoptosis of human cancer cells. *J. Appl. Microbiol.* 117 (2), 498–508. doi: 10.1111/jam.12531

Nieves-Ramírez, M. E., Partida-Rodríguez, O., Moran, P., Serrano-Vázquez, A., Pérez-Juárez, H., Pérez-Rodríguez, M. E., et al. (2021). Cervical squamous intraepithelial lesions are associated with differences in the vaginal microbiota of Mexican women. *Microbiol. spectrum* 9 (2), e0014321. doi: 10.1128/Spectrum.00143-21

Norenhag, J., Du, J., Olovsson, M., Verstraelen, H., Engstrand, L., and Brusselaers, N. (2020). The vaginal microbiota, human papillomavirus and cervical dysplasia: a systematic review and network meta-analysis. *BJOG an Int. J. obstetrics gynaecol* 127 (2), 171–180. doi: 10.1111/1471-0528.15854

Oliveira, G. H., and Schirger, A. (2003). Images in clinical medicine. page kidney. *New Engl. J. Med.* 348 (2), 129. doi: 10.1056/NEJMicm020037

Palma, E., Recine, N., Domenici, L., Giorgini, M., Pierangeli, A., and Panici, P. B. (2018). Long-term lactobacillus rhamnosus BMX 54 application to restore a balanced vaginal ecosystem: a promising solution against HPV-infection. *BMC Infect. dis* 18 (1), 13. doi: 10.1186/s12879-017-2938-z

Petrova, M. I., van den Broek, M., Balzarini, J., Vanderleyden, J., and Lebeer, S. (2013). Vaginal microbiota and its role in HIV transmission and infection. *FEMS Microbiol. Rev.* 37 (5), 762–792. doi: 10.1111/1574-6976.12029

Piyathilake, C. J., Ollberding, N. J., Kumar, R., Macaluso, M., Alvarez, R. D., and Morrow, C. D. (2016). Cervical microbiota associated with higher grade cervical intraepithelial neoplasia in women infected with high-risk human papillomaviruses. *Cancer Prev. Res. (Philadelphia Pa).* 9 (5), 357–366. doi: 10.1158/1940-6207.CAPR-15-0350

Pyeon, D., Pearce, S. M., Lank, S. M., Ahlquist, P., and Lambert, P. F. (2009). Establishment of human papillomavirus infection requires cell cycle progression. *PLos pathogens* 5 (2), e1000318. doi: 10.1371/journal.ppat.1000318

Qingqing, B., Jie, Z., Songben, Q., Juan, C., Lei, Z., and Mu, X. (2021). Cervicovaginal microbiota dysbiosis correlates with HPV persistent infection. *Microbial pathogenesis* 152, 104617. doi: 10.1016/j.micpath.2020.104617

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. United States America* 108 Suppl 1 (Suppl 1), 4680–4687. doi: 10.1073/pnas.1002611107

Ritu, W., Enqi, W., Zheng, S., Wang, J., Ling, Y., and Wang, Y. (2019). Evaluation of the associations between cervical microbiota and HPV infection, clearance, and persistence in cytologically normal women. *Cancer Prev. Res. (Philadelphia Pa).* 12 (1), 43–56. doi: 10.1158/1940-6207.CAPR-18-0233

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ.* 4, e2584. doi: 10.7717/peerj.2584

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., et al. (2014). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome.* 2 (1), 4. doi: 10.1186/2049-2618-2-4

Romero-Morelos, P., Bandala, C., Jiménez-Tenorio, J., Valdespino-Zavala, M., Rodríguez-Esquivel, M., Gama-Ríos, R. A., et al. (2019). Vaginosis-associated bacteria and its association with HPV infection. *Med clinica* 152 (1), 1–5. doi: 10.1016/j.medcle.2018.11.005

Schiffman, M., Doorbar, J., Wentzensen, N., de Sanjosé, S., Fakhry, C., Monk, B. J., et al. (2016). Carcinogenic human papillomavirus infection. *Nat. Rev. Dis. primers* 2, 16086. doi: 10.1038/nrdp.2016.86

Seo, S. S., Oh, H. Y., Lee, J. K., Kong, J. S., Lee, D. O., and Kim, M. K. (2016). Combined effect of diet and cervical microbiome on the risk of cervical intraepithelial neoplasia. *Clin. Nutr. (Edinburgh Scotland)* 35 (6), 1434–1441. doi: 10.1016/j.clnu.2016.03.019

Serrano, M. G., Parikh, H. I., Brooks, J. P., Edwards, D. J., Arodz, T. J., Edupuganti, L., et al. (2019). Racioethnic diversity in the dynamics of the vaginal microbiome during pregnancy. *Nat. Med.* 25 (6), 1001–1011. doi: 10.1038/s41591-019-0465-8

Shannon, B., Yi, T. J., Perusini, S., Gajer, P., Ma, B., Humphrys, M. S., et al. (2017). Association of HPV infection and clearance with cervicovaginal immunology and the vaginal microbiota. *Mucosal Immunol.* 10 (5), 1310–1319. doi: 10.1038/mi.2016.129

Srinivasan, S., Liu, C., Mitchell, C. M., Fiedler, T. L., Thomas, K. K., Agnew, K. J., et al. (2010). Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLos One* 5 (4), e10197. doi: 10.1371/journal.pone.0010197

Stoyancheva, G., Marzotto, M., Dellaglio, F., and Torriani, S. (2014). Bacteriocin production and gene sequencing analysis from vaginal lactobacillus strains. *Arch. Microbiol.* 196 (9), 645–653. doi: 10.1007/s00203-014-1003-1

Usyk, M., Zolnik, C. P., Castle, P. E., Porras, C., Herrero, R., Gradissimo, A., et al. (2020). Cervicovaginal microbiome and natural history of HPV in a longitudinal study. *PLos pathogens* 16 (3), e1008376. doi: 10.1371/journal.ppat.1008376

Verhoeven, V., Renard, N., Makar, A., Van Royen, P., Bogers, J. P., Lardon, F., et al. (2013). Probiotics enhance the clearance of human papillomavirus-related cervical lesions: a prospective controlled pilot study. *Eur. J. Cancer Prev. Off. J. Eur. Cancer Prev. Organisation (ECP).* 22 (1), 46–51. doi: 10.1097/CEJ.0b013e328355ed23

Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., et al. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. pathology* 189 (1), 12–19. doi: 10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73 (16), 5261–5267. doi: 10.1128/AEM.00062-07

Wang, K. D., Xu, D. J., Wang, B. Y., Yan, D. H., Lv, Z., and Su, J. R. (2018). Inhibitory effect of vaginal lactobacillus supernatants on cervical cancer cells. *Probiotics antimicrobial proteins* 10 (2), 236–242. doi: 10.1007/s12602-017-9339-x

Wilkinson, E. J., Cox, J. T., Selim, M. A., and O'Connor, D. M. (2015). Evolution of terminology for human-papillomavirus-infection-related vulvar squamous intraepithelial lesions. *J. lower genital tract disease* 19 (1), 81–87. doi: 10.1097/LGT.0000000000000049

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Tian Xiao Huang,
Nanchang University, China
Chao-an Long,
Huazhong Agricultural University, China

*CORRESPONDENCE
Bin Zhang
zhangbin2919@163.com
Xiaoyu Wu
xiaoyuwu@jxau.edu.cn

†These authors have contributed equally to this work

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Physiological and transcriptome analysis of *Candida albicans* in response to X33 antimicrobial oligopeptide treatment

Qunlin Lu[1,2,3†], Yuanxiu Wang[1,2,3†], Xing Liao[1,2,3], Fu Zhou[1,2,3], Bin Zhang[1,2,3*] and Xiaoyu Wu[1,2,3*]

[1]College of Bioscience and Bioengineering, Jiangxi Agriculture University, Nanchang, China, [2]Jiangxi Engineering Laboratory for the Development and Utilization of Agricultural Microbial Resources, Jiangxi Agriculture University, Nanchang, China, [3]Collaborative Innovation Center of Postharvest Key Technology and Quality Safety of Fruits and Vegetables in Jiangxi Province, Jiangxi Agriculture University, Nanchang, China

Introduction: *Candida albicans* is an opportunistic pathogenic fungus, which frequently causes systemic or local fungal infections in *humans*. The evolution of its drug-resistant mutants necessitate an urgent development of novel antimicrobial agents.

Results: Here, we explored the antimicrobial activity and inhibitory mechanisms of X33 antimicrobial oligopeptide (X33 AMOP) against *C. albicans*. The oxford cup test results showed that X33 AMOP had strong inhibitory activity against *C. albicans*, and its MIC and MFC were 0.625 g/L and 2.5 g/L, respectively. Moreover, SEM and TEM showed that X33 AMOP disrupted the integrity of cell membrane. The AKP, ROS, H_2O_2 and MDA contents increased, while the reducing sugar, soluble protein, and pyruvate contents decreased after the X33 AMOP treatment. This indicated that X33 AMOP could damage the mitochondrial integrity of the cells, thereby disrupting the energy metabolism by inducing oxidative stress in *C. albicans*. Furthermore, transcriptome analysis showed that X33 AMOP treatment resulted in the differential expression of 1140 genes, among which 532 were up-regulated, and 608 were down-regulated. These DEGs were related to protein, nucleic acid, and carbohydrate metabolism, and their expression changes were consistent with the changes in physiological characteristics. Moreover, we found that X33 AMOP could effectively inhibit the virulence attributes of *C. albicans* by reducing phospholipase activity and disrupting hypha formation.

Discussion: These findings provide the first-ever detailed reference for the inhibitory mechanisms of X33 AMOP against *C. albicans* and suggest that X33 AMOP is a potential drug candidate for treating *C. albicans* infections.

KEYWORDS

X33 antimicrobial oligopeptide, *Candida albicans*, antimicrobial mechanism, transcriptomics, cell damage

Highlights

- X33 AMOP have a negative impact on the growth of C. albicans.
- X33 AMOP suppressed morphological transformation of C. albicans.
- X33 AMOP interfered with the intracellular proteins and reducing sugar synthesis of C. albicans.
- X33 AMOP caused genetic information delivery change, destruction of cell wall and cell membrane integrity.
- X33 AMOP accelerated the production of ROS, MDA and H2O2, and induced oxidative damage.

1 Introduction

Fungal infections have become serious threats to human health, with a mortality rate of up to 45% (Rauseo et al., 2020). *C. albicans* is an opportunistic human fungal pathogen, which can change from yeast to hyphal growth forms and is widely distributed on the skin and the mucous membrane of the oral cavity and vagina (Ghannoum et al., 2010). Though a human commensal, *C. albicans* infects the body when the host's immunity is reduced, causing superficial and invasive infections (Ruhnke, 2006). In extreme cases, invasive candidiasis causes a serious health threat to patients (Dimopoulos et al., 2007). Among the *Candida* species, *C. albicans* is the most widely distributed species, causing life-threatening systemic blood infections in immunocompromised humans. For example, candidemia caused by *C. albicans*, has been identified as the third or fourth most cause of bloodstream infections and the first cause of severe infections in the intensive care units (Pappas et al., 2016). *C. albicans* invasive infections frequently result in candidemia and invasive candidiasis, leading to 20 - 34% and 45 - 70% of mortality rates in newborns and adults, respectively (Antinori et al., 2016; Fu et al., 2017). Currently, various drugs, including polyenes (amphotericin B, nystatin), azoles (fluconazole, itraconazole), echinocandins (caspofungin, micafungin), etc., have been discovered and used as clinical treatment of various fungal infections. Despite exerting a bacteriostatic effect against *C. albicans* by destroying its cell wall or cell membrane (Hoehamer et al., 2010), these drugs are toxic to the liver and kidney, expensive, and cannot contain the drug-resistant strains. Therefore, developing novel antimicrobial agents that are effective and safe is crucial for treating fungal infections.

Antimicrobial peptides (AMPs) are 10 - 50 amino acid residues containing functional peptides with defensive activities for human immunity. Thus, AMPs are considered promising drugs due to their antibacterial, antifungal, antivirus, and antitumor properties (Huan et al., 2020). AMPs are widely produced by prokaryotes, unicellular bacteria (non-filamentous bacteria), and eukaryotic fungi capable of protecting their host from pathogens. *Streptomyces* produce various products with important biological activities, which can serve as antimicrobial peptides, antibiotics, immunosuppressants, anticancer drugs, etc., (Berdy, 2012). Notably, secondary metabolites produced by *Streptomyces* play a key role in treating infectious diseases caused by *C. albicans* (Sivalingam et al., 2019). For example, ϵ-polylysine, produced by *Streptomyces albus*, exerts a wide range of antimicrobial activities against yeast and is widely applied in food, medical, and biopharmaceutical industries (Shi et al., 2015). Moreover, *Streptomyces lavendulae* X33 (isolated in our previous study) secretes X33 antimicrobial oligopeptide (X33 AMOP), which strongly inhibits *Penicillium digitatum* (Lin et al., 2020). The recent advancement in transcriptomic analysis has greatly enhanced the ability to analyze the inhibition mechanisms of antimicrobial peptides against pathogens. For example, transcriptome analysis has been used to explore the changes in *C. albicans* target areas after treatment with MAF-1 (Wang et al., 2017).

In this study, we found that X33 AMOP also exhibits preferable antimicrobial effects on *C. albicans*. We used transcriptome and physiological analysis methods to investigate the bacteriostatic mechanism of X33 AMOP against *C. albicans*. The findings of this study provide a theoretical basis for antifungal infection drug research and development.

2 Materials and methods

2.1 Strains and reagents

According to the previous method, X33 AMOP was isolated from the fermentation supernatant of *S. lavendulae* X33 (stored in the typical culture preservation center of China, No. CCTCC M2013163) (Lin et al., 2020). *C. albicans* ATCC 12322 was obtained from Jiangxi microbial strain collection and management center. *C. albicans* was stored in glycerol at - 80℃, and then streaked onto YPD agar (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar). A single colony was then inoculated in YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) to obtain fresh yeast cells. The cells were resuspended with RPMI 1640 and adjusted to 10^7 CFU/mL to prepare cell suspension for further analysis.

Abbreviations: X33 AMOP, X33 antimicrobial oligopeptide; SEM, scanning electron microscope; TEM, transmission electron microscope; AKP, alkaline phosphatase; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; AMPs, antimicrobial peptides; MAF-1, Musca domestica antifungal peptide-1; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; RT-PCR, reverse transcription-PCR; qRT-PCR, quantitative real-time PCR; DEGs, differentially expressed genes; HMDS, hexamethyldisilazane; FDA, fluorescein diacetate; PI, propidium iodide; TCA, tricarboxylic acid; Pz, the activity of phospholipase; SD, standard deviation; DCFH-DA, 2', 7'-dichlorofluorescein diacetate; DCF, dichlorofluorescein; PM, plasma membrane; Q20, the percentage of bases with sequencing quality of more than 99% in the total base; Q30, the sequencing quality of more than 99.9% in the total base; PCA, principal component analysis; PC1, principal component 1; PCA2, principal component 2; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; HSAF, heat-stable activity factor is a polycyclic tetramate macrolactam; CEK, extracellular signal-regulated kinase.

2.2 Evaluation of X33 AMOP antifungal activity

To evaluate the inhibitory effect of X33 AMOP on the growth of *C. albicans*, the minimum inhibitory concentration (MIC) of X33 AMOP was determined according to a previous method with slight modification (Sun et al., 2022). The concentration of X33 AMOP was half diluted from 20 g/L to 0.039 g/L by continuous dilution method, and sterile water was used as a control.

The activity of the X33 AMOP against *C. albicans* was tested using the oxford cup method. Briefly, the cell suspension was evenly spread on the YPD agar under sterile conditions, and the oxford cups were placed in the center of the medium. Different concentrations of X33 AMOP (1/4 MIC, 1/2 MIC and MIC) and sterile water (control) were added (200 μL each) to each cup, and plates were subsequently cultivated at 37℃ for 24 h. The diameter of the antibacterial circle was measured with a vernier caliper using the cross method and was stated as mean ± standard error. These tests were repeated three independent times.

The time sterilization curve of X33 AMOP against *C. albicans* was drawn by sampling at different time points. The X33 AMOP with different concentrations were added, and sterile water as the control. The conical flasks were incubated at 37℃ with 200 r/min shaking to 12 h. Fermentation broth was taken at 2 h intervals to measure the absorbance. The growth curve was plotted as absorbance at 560 nm against time interval.

2.3 RNA sequencing and RT-PCR

The cells were added to YPD broth and cultured at 37℃ for 4 h with X33 AMOP at 0.625 g/L for the E group and 0 g/L for the C group. Thereafter, cells were harvested, washed several times with sterile water and quickly frozen in liquid nitrogen for total RNA extraction. The RNA was extracted using TRIzol® Reagent following the manufacturer's instructions, and the subsequent RNA-seq and RT-PCR analysis was processed as described previously (Lin et al., 2020). All gene-specific primers used in this study are listed in the supporting materials. The fold change of each gene in the experiment group (E group) compared with the control group (C group) was tested in three replicates and was calculated with reference to the internal reference genes RDN18 using the $2^{-\Delta\Delta Ct}$ algorithm (Wang et al., 2017).

2.4 Electron microscopy of *C. albicans* cells

C. albicans cells were incubated with X33 AMOP (MIC) at 37°C for 0, and 4 h (Ma et al., 2020). Thereafter, the cells were fixed with 2.5% glutaraldehyde and dehydrated with 50%, 60%, 70%, 80%, 90%, 95% and 100% ethanol gradients, followed by drying using 98% hexamethyldisilazane (HMDS). The samples were then coated with gold and imaged with the TESCAN vega 3 LMU scanning electron microscope (SEM).

Since the changes in internal cellular morphology can be used to evaluate the antifungal effect of X33 AMOP on *C. albicans*, the cells were incubated with X33 AMOP (MIC) in the YPD medium at 37°C for 0, 4, and 12 h (Su et al., 2020). The cells were then fixed with 2.5% glutaraldehyde and 1% osmic acid solution at 4°C. After dehydrating with 50%, 60%, 70%, 80%, 90%, 95% and 100% ethanol, the samples were treated with an embedding agent and acetone. The samples were then sliced in an ultra-thin microtome and stained with lead citrate solution, followed by a saturated solution of uranyl acetate and 50% for 10 min each. Thereafter, the stained samples were observed by transmission electron microscope (TEM).

2.5 Determination of alkaline phosphatase content

To explore the inhibitory mechanism of X33 AMOP against *C. albicans*, we measured the activity of alkaline phosphatase (AKP), an indicator frequently used to evaluate the damage or integrity of the cell wall. The AKP activities of the different concentrations of X33 AMOP treatment were determined using a spectrophotometer and an AKP kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions (Song et al., 2018). The cells were cultured with different X33 AMOP concentrations at 37°C for 8 h, and their supernatants were collected every 4 h to measure the AKP content.

2.6 Sorbitol assay

Sorbitol, an osmotic pressure stabilizer, was added into the medium according to a previous method, and its MIC changes were measured before and after addition (Leite et al., 2014). Thereafter, MIC and 1/2 MIC cultures were serially diluted and spread on YPD agar. The plates were incubated at 37°C for 36 h, and the number of *C. albicans* colonies on each plate was recorded.

2.7 Permeability of cell membranes

The cell membrane is an important physiological structure that maintains the intracellular environment stability of cells. When the cell membrane is destroyed, its barrier function is damaged, releasing the intracellular substances into the extracellular environment. Membrane permeability was measured by the FDA-PI two-color fluorescence method (Ross et al., 1989). Different concentrations of X33 AMOP and sterile water were added to the cell suspension and cultured on a shaker incubator at 37°C and 200 r/min for 12 h. The cell solution was placed on the slide and stained with 2 μL fluorescein diacetate (FDA), followed by 5 μL propidium iodide (PI) for 8 min each. The excess dye was washed off, and the cells were observed under an inverted fluorescence microscope (Nikon).

2.8 Determination of oxidative stress parameters in *C. albicans*

To explore the oxidative damage of *C. albicans* caused by X33 AMOP treatment, we measured the level of the reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) according to

the previous study (Ren et al., 2020). Briefly, the cells were exposed to 1/4 MIC, 1/2 MIC and MIC of X33 AMOP for 2, 4, and 8 h. Sterile water was used as the control. The cells were resuspended in PBS to 10^6 CFU/mL, and the ROS was examined by the reactive oxygen species assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the instructions. The H_2O_2 content of the different concentrations of X33 AMOP treatments was measured using a hydrogen peroxide kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the instructions. Conversely, the thiobarbituric acid method was used to measure the MDA content of the cells.

2.9 Effect of X33 AMOP on glucose-stimulated acidification of the external medium

After incubation for 10 h at 37°C on a shaker at 200 r/min, *C. albicans* cells were collected, washed, and resuspended in sterile water. Added X33 AMOP, with sterile water as the control, and then incubated at 37°C for 2 h. Then, washed with sterile water to remove the antibacterial peptide and suspended in 10% $C_6H_{12}O_6$ solution, and the extracellular pH was measured at 0, 1, 2,4, and 6 h respectively (da Silva Neto et al., 2020).

2.10 Determination of pyruvic acid, intracellular reducing sugar, and soluble protein contents

Pyruvic acid is a crucial metabolite involved in various synthesis and catabolism pathways, including amino acid synthesis, glycolysis, gluconeogenesis pathway, and tricarboxylic acid cycle (TCA cycle). Pyruvic acid content was measured according to a previous method with slight modifications (Zou et al., 2015). Briefly, the samples were homogenized in 8% trichloroacetic acid, centrifuged at 6000 r/min for 10 min, and the supernatant was collected for pyruvic acid content determination. Reducing sugars and soluble proteins are indispensable cellular components necessary for maintaining life activities. Furthermore, soluble protein content was determined using a soluble protein detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions (Chen et al., 2020a).

2.11 Determination of phospholipase activity and morphology transformation

Extracellular phospholipase activities were measured using a previous method with some modifications (Srivastava et al., 2018). Briefly, 1 μL of the cells was incubated on the egg yolk solid medium at 37°C for 36 h in the absence or presence of the different concentrations of X33 AMOP. The dense white zone of precipitation around the colonies showed the presence of phospholipase, whose activity (Pz) was calculated by the formula: colony diameter/(colony diameter + precipitation zone diameter). The effect of X33 AMOP on the yeast morphology of *C. albicans* was also assessed (Priya and Pandian, 2020). Different concentrations of X33 AMOP were added to the cells, and the cultures were shaken for 4, 8 and 12 h, followed by observing their morphological changes under a light microscope (Olympus).

2.12 Statistical analysis

Data were presented as average ± standard deviation (SD) of the three independent experiments. One-way analysis of variance (ANOVA) and Duncan's *post hoc* test were performed with a significant p-value of < 0.05 using the SPSS statistical software to analyze the significant difference between the values of control and treated samples.

3 Results

3.1 X33 AMOP inhibited the growth of *C. albicans*

The antimicrobial activity of X33 AMOP was performed by growth inhibition test which indicated that the MIC and MFC were 0.625 g/L and 2.5 g/L, respectively (Table 1). Additionally, the oxford cup test results showed that the antimicrobial diameter formed by 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP were 16.31 ± 0.39, 18.49 ± 0.38, and 20.74 ± 0.47 mm, respectively (Figures 1A, B). Thus, the inhibitory effect of X33 AMOP against *C. albicans* increased in a dose-dependent manner. As shown in Figure 1C, X33 AMOP strongly inhibited the growth of *C. albicans*, and the inhibitory effect of MIC was higher than in the other groups. For the control group, *C. albicans* formed a normal growth curve and had an OD_{560} of 2.12 ± 0.10 at 12 h in the absence of X33 AMOP. However, when treated with 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP, the growth of *C. albicans* exhibited a continuous decline and had OD_{560} values of 0.73 ± 0.03, 0.46 ± 0.01, and 0.37 ± 0.01 at 12 h, respectively. These results suggested a strong inhibitory effect of X33 AMOP on the growth of *C. albicans*.

3.2 X33 AMOP caused cell wall damage in *C. albicans*

As shown in Figure 2A, the AKP activities were 0.84 ± 0.09 ($p = 0.16 > 0.05$), 1.03 ± 0.13 ($p < 0.05$), and 1.60 ± 0.11 ($p < 0.001$) U/L

TABLE 1 The MIC and MFC determination.

Concentrations (mg/mL)	20	10	5	2.5	1.25	0.625	0.3125	0.15625	0.078125	0.039	Control
Clear (-, +) a	–	–	–	–	–	–	+	+	+	+	+
Colony (-, +) b	–	–	–	+	+	+	–	–	–	–	+

a Minimum inhibitory concentration, b Minimum fungicidal concentration.

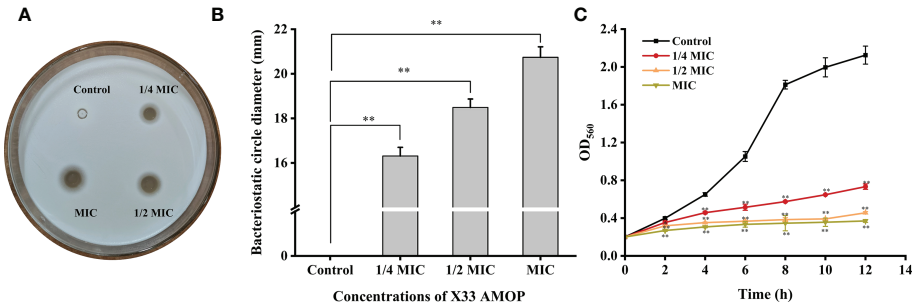

FIGURE 1
Effects of X33 AMOP on (*C*) *albicans*. **(A, B)** The diameters of the bacteriostatic circle of different X33 concentrations AMOP against (*C*) *albicans*. **(C)** The growth curves of (*C*) *albicans*. The different colors show the growth curves at different X33 AMOP concentrations. *$p < 0.05$, **$p < 0.01$.

when the cells were treated with 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP for 4 h, respectively, which were higher than that of the control group (0.64 ± 0.05 U/L). Compared with the control group, the AKP activities of 1/4 MIC, 1/2 MIC, and MIC of the X33 AMOP treatment increased by 0.59-, 1.05-, and 1.61-folds during the 8 h incubation with *C. albicans*, respectively. To further confirm this observation, we added sorbitol, a cell wall protector to the treatment groups. The logic was that the protective effect of sorbitol should be reflected by the increasing MIC values (Svetaz et al., 2007). Nonetheless, the MIC of X33 AMOP did not change when 0.8 M sorbitol was added to the incubation system, but the number of viable cells in the 1/2 MIC and MIC of X33 AMOP treatment groups reached $9.30*10^6 \pm 4.24*10^5$ and $1.92*10^5 \pm 9.31*10^3$ CFU/mL, respectively. These values were significantly higher than that of the control groups ($1.04*10^6 \pm 0.28*10^5$ and $1.44*10^4 \pm 0.85*10^3$ CFU/mL) in the absence of sorbitol Figure 2B. Therefore, we concluded that X33 AMOP could initiate cell wall damage in *C. albicans*.

3.3 X33 AMOP induced membrane permeability changes in *C. albicans*

Viable cells with integrated membrane exhibit green fluorescence, while the damaged cell membrane exhibits a red fluorescent under the fluorescence microscope. In this study, the control group cells exhibited a robust green fluorescence and a weak red fluorescence. However, the red fluorescence gradually increased, while the green fluorescence decreased after the treatment with 1/4 MIC, 1/2 MIC and MIC of X33 AMOP Figure 3A. Microscopy imaging of *C. albicans* using the scanning microscope indicated that the cells changed from complete, smooth, plump, and regulated morphology to deformed and wrinkled interconnection morphology after the treatment with X33 AMOP (Figure 3B). When the cell membrane is destroyed, macromolecular substances such as proteins, and reducing sugars, originally existing in the cell are leaked out of the cell. We found that the membrane leakage of protein reached 32.77 ± 0.41, 34.50 ± 0.77, 51.43 ± 1.82 mg/L after treatment with 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP, respectively. These values were 0.45-, 0.53-, and 1.28-fold higher than that of the control group (22.56 ± 0.32 mg/L), respectively (Figure 3C). Similarly, the leakage of reducing sugars increased by 0.89-, 1.85- and 3.10-fold after treatment with 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP, respectively (Figure 3D). These results showed that X33 AMOP destroyed the integrity of *C. albicans* cell membrane, resulting in the leakage of reducing sugars and proteins.

3.4 X33 AMOP induced oxidative stress reaction in *C. albicans*

The accumulation of ROS has been shown to play a crucial role in programmed cell death of yeast and could therefore be an element of cell membrane damage (Sun et al., 2022). As shown in Figure 4A, ROS accumulation in *C. albicans* increased significantly after the treatment

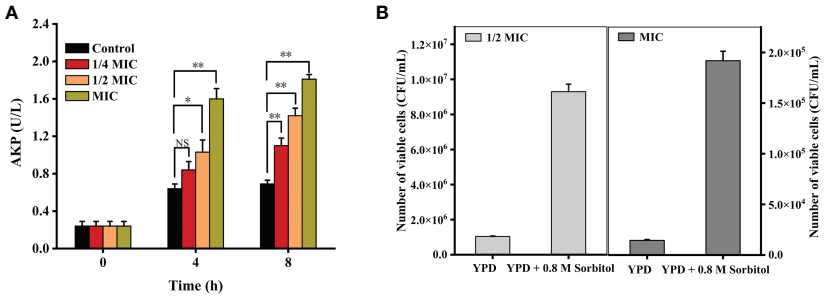

FIGURE 2
Effects of the different X33 AMOP concentrations on (*C*) *albicans* cell wall integrity. **(A)** Alkaline phosphatase activity of the different X33 AMOP concentrations on (*C*) *albicans*. The different colors show the growth of the different X33 AMOP concentrations. **(B)** The cell vitality of (*C*) *albicans*. *$p < 0.05$, **$p < 0.01$, NS: $p > 0.05$.

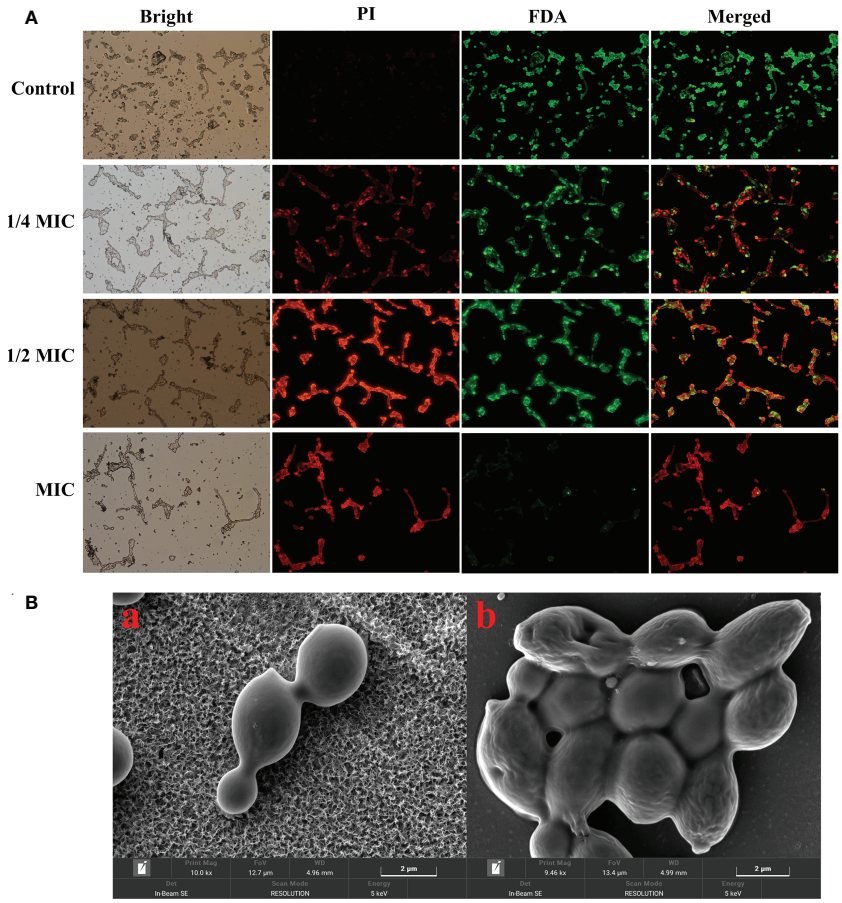

FIGURE 3
Effects of X33 AMOP on the cell membrane permeability of (*C*) *albicans*. **(A)** Fluorescent microscopy of (*C*) *albicans* cells. The green color represents viable cells, while the red color represents dead cells. **(B)** Scanning electron microphotographs of (*C*) *albicans* cells subjected to X33 AMOP treatment at the MIC for (a) 0 h, (b) 4 h.

with X33 AMOP. After a 2 h treatment with 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP, ROS accumulation in *C. albicans* increased by 0.19-, 0.30-, and 0.86-fold compared with the control group. However, the increment reached 3.14-, 3.56- and 5.88- fold when *C. albicans* cells were treated with 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP for 8 h, respectively. ROS consist of superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and nitric oxide. Lipid peroxidation promotes the formation of peroxyl radicals, which destroy the cell membrane stability, resulting in induced cell death (Patriota et al., 2016). To further explore the oxidative stress in *C. albicans*, we measured the content of H_2O_2. As shown in Figure 4B, the H_2O_2 contents were 3.37 ± 0.01, 3.95 ± 0.01, and 4.51 ± 0.08 mmol/g, representing 13%, 32%, and 51% increase compared to the control group (2.98 ± 0.09 mmol/g), after 8 h treatment with 1/4 MIC, 1/2 MIC, and MIC, respectively. Since ROS production damages the cell membrane, resulting in lipid peroxidation and MDA generation, we also measured the MDA contents to explore the oxidative stress effects on *C. albicans*. We found that the MDA contents in *C. albicans* were 4.29 ± 0.25, 4.93 ± 0.74, and 6.34 ± 0.27 μmol/g, which were 19%, 37%, and 76% higher than that of the control group (3.61 ± 0.14 μmol/g), under 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP treatment, respectively (Figure 4C). Plasma membrane (PM) H^+-ATPase is a primary pump that plays various roles in cell metabolism and maintaining transmembrane H^+ electrochemical gradient (da Silva Neto et al., 2020). Upon their induction with glucose, normal cells activate the proton pump on their membranes and secrete acid to the culture medium to maintain intracellular homeostasis and osmotic pressure stability. In this study, the external medium acidification of *C. albicans* was inhibited by X33 AMOP after their induction with glucose (Figure 4D). When MIC of X33 AMOP was added to the medium, the pH value decreased by 0.86 after induction for 6 h, while that of the control group reduced by 1.14. This showed that the acidification ability of the control group was stronger than that of the treatment group. Thus, we concluded that X33 AMOP could induce oxidative stress in *C. albicans*.

3.5 X33 AMOP triggered the cellular content changes in *C. albicans*

The transmission electron microscopy results suggested that the *C. albicans* cells treated with X33 AMOP were seriously damaged, their contents were leaked out, and their organelles were agglomerated. However, the control groups exhibited dense, uniform, regular, and clearly visible cellular organelles (Figure 5A). What's more, to evaluate the cellular content changes in *C. albicans*

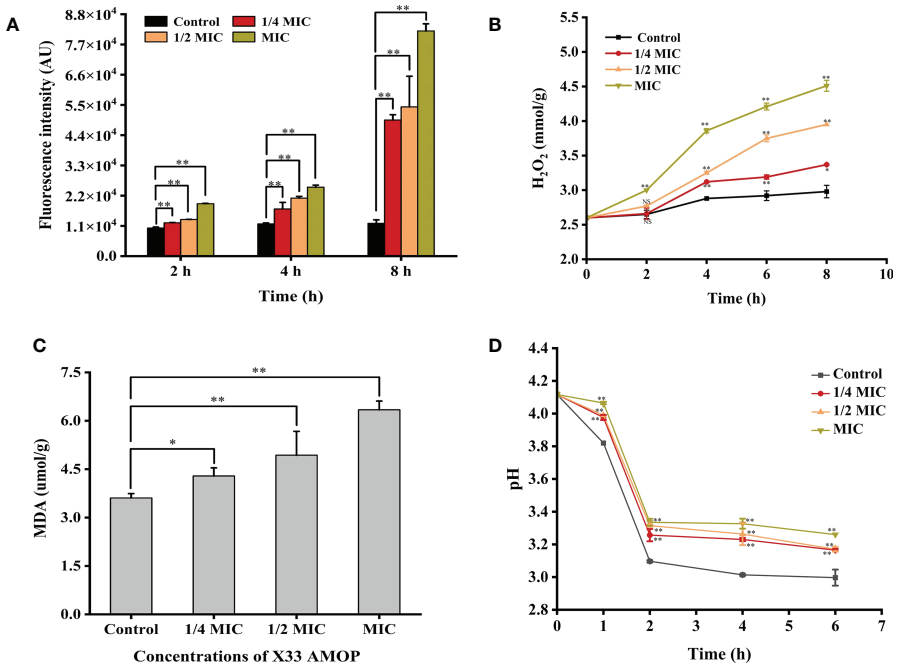

FIGURE 4
Effects of the different X33 AMOP concentrations on the oxidative stress level in (*C*) *albicans*. **(A)** The fluorescence intensity of dichlorofluorescein (fluorescence intensity is proportional to ROS). **(B)** The H_2O_2 contents at the different X33 AMOP concentrations. **(C)** The MDA contents at the different X33 AMOP concentrations. **(D)** Effects of X33 AMOP on the glucose-dependent acidification of (*C*) *albicans* culture medium. The different colors show different concentrations of X33 AMOP. *$p < 0.05$, **$p < 0.01$, NS: $p > 0.05$.

treated with X33 AMOP, we measured the intracellular pyruvic acid content using the 2,4-dinitrophenylhydrazine method. As shown in Figure 5B, the intracellular pyruvic acid content of the control group was 57.14 ± 1.88 μg/g, while that of the 1/4 MIC, 1/2 MIC, and MIC treatment groups were 50.25 ± 3.26, 46.46 ± 2.85, and 32.30 ± 0.38 μg/g, respectively. This showed that X33 AMOP induced metabolism disturbance in *C. albicans*. We also evaluated the intracellular soluble protein content to further evaluate the cellular content changes induced by the X33 AMOP treatment in *C. albicans*. We found that the soluble protein contents of *C. albicans* were 0.89 ± 0.01, 0.83 ±

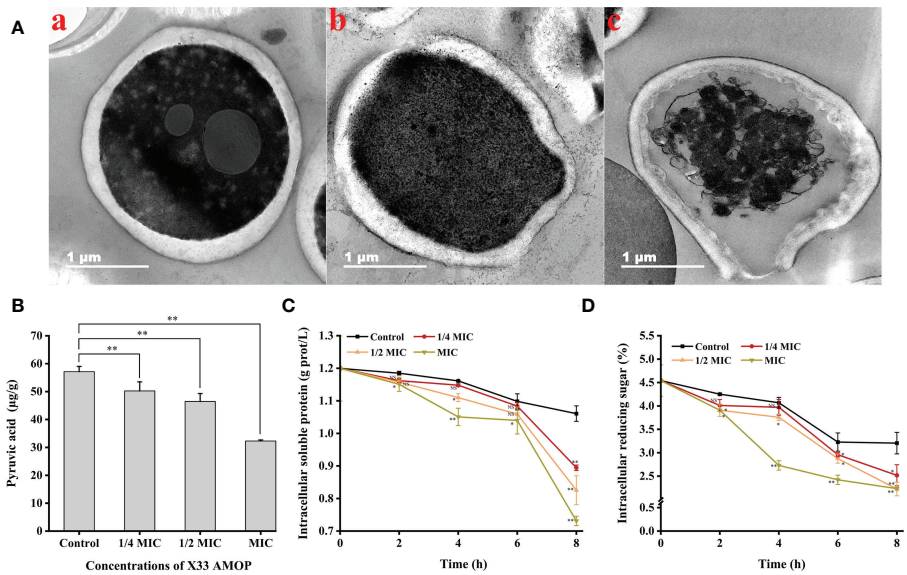

FIGURE 5
Effects of the different X33 AMOP concentrations on the cellular contents of (*C*) *albicans*. **(A)** Transmission electron microscopy of *(C) albicans* cells treated with X33 AMOP at the MIC for (a) 0 h, (b) 4 h, and (c) 12 h. **(B)** The pyruvic acid content of (*C*) *albicans* at the different X33 AMOP concentrations. **(C)** The soluble protein contents of (*C*) *albicans* at the different X33 AMOP concentrations. **(D)** The reducing sugar content of (*C*) *albicans* at the different X33 AMOP concentrations. The different colors show different concentrations of X33 AMOP. *$p < 0.05$, **$p < 0.01$, NS: $p > 0.05$.

0.04, and 0.73 ± 0.01 g/L after the 8 h treatment with 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP, respectively (Figure 5C). These values were lower than that of the control group (1.06 ± 0.02 g/L) (Figure 5C). Similarly, the reducing sugar content also reduced to 2.52 ± 0.23, 2.22 ± 0.14, and 2.23 ± 0.03% when *C. albicans* were treated with 1/4 MIC, 1/2 MIC, and MIC of X33 AMOP, respectively, were lower than the control group (3.20 ± 0.23%) (Figure 5D). These results indicated that X33 AMOP treatment disrupts the anabolism and integrity of *C. albicans* cellular components.

3.6 Transcriptome analysis of *C. albicans* in response to X33 AMOP treatment

Here, we used transcriptome analysis of gene expression to comprehensively construct the inhibitory model of X33 AMOP against *C. albicans*. The quality assessment results showed that the percentage of bases with a sequencing quality of 99% (Q20) was higher than 85%, while the sequencing quality of 99.9% (Q30) was higher than 80%. Moreover, the error rates of six samples were less than 0.1%, indicating that sequencing samples were not contaminated and met the requirements for transcriptional analysis (Table 2). The biological repeat correlation analysis was performed to compare the correlation of C (control group) and E (X33 AMOP treatment group) samples (Figure 6A). We found that the C and E samples had closer correlation values, showing a higher similarity of gene expression between the samples. The principal component analysis (PCA) reflects intra-group repeatability and inter-group difference by clustering the samples into components. The replicates of groups C and E clustered well, indicating consistency between the biological replicates. The PC1 and PC2 clusters constituted 57.9% and 23.5% of the samples, respectively (Figure 6B). Among the 6045 expressed genes, 1140 met the screening conditions of the differentially expressed genes (p-adjust < 0.05 & |log2FC| ≥ 1), among which 532 were up-regulated, and 608 were down-regulated (Figure 6C). To verify the accuracy of RNA-seq data, we selected ten DEGs randomly for validation using RT-qPCR (Primer sequences were shown in Table S1). Consistent with the RNA-seq results, the RT-qPCR results showed that the expression of *MDH1*, *HBR2*, and *IDP2* were upregulated, while *SOD3*, *MET14*, *ADH2*, *LEU4*, *CYS3*, *CEK1*, and *RPO21* were downregulated tests (Figure 6D). Thus, these results proved the reliability of the RNA-seq data.

Furthermore, we conducted Gene Ontology (GO) term and KEGG enrichment analyses to systematically explore the biological functions and pathways of the obtained DEGs. The GO term analysis indicated that 518 genes were divided into three-term types and 152 hierarchies. The up-regulated genes were mainly involved in the protein, nucleic acid, and carbohydrate metabolism, while the up-regulated genes were enriched in nucleosome, DNA packaging complex and protein-DNA complex. The molecular function enrichment analysis suggested that the up-regulated genes were involved in transmembrane transporter, symporter and protein dimerization activities. Moreover, the biological process enrichment analysis suggested that the up-regulated genes were involved in transmembrane transport and nucleotide-sugar biosynthetic process (Figure 7A). However, the down-regulated genes were enriched in the anchored components and extracellular regions of the membrane. According to the biological process enrichment analysis, these down-regulated genes were involved in interspecies interaction between organisms, pathogenesis, sulfur compound metabolic process, and biological adhesion. Therefore, X33 AMOP induced expression changes in various genes related to various metabolism and cellular processes in *C. albicans* (Figure 7B). The KEGG pathway analysis results showed that 335 genes were classified into 89 pathways, among which the up-regulated genes were significantly enriched in nine ($p < 0.05$). Among these, the arginine biosynthesis pathway had six, while glyoxylate and dicarboxylate metabolism had seven up-regulated genes. Eight up-regulated genes were enriched in cysteine and methionine metabolism, three were enriched in pentose and glucuronate interconversions, and six were enriched in arginine and proline metabolism pathways. Among them, the top three enriched pathways were arginine biosynthesis, glyoxylate and dicarboxylate metabolism and cysteine and methionine metabolism (Figure 7C). The down-regulated genes were significantly enriched ($p < 0.05$) in eight pathways, including mitogen-activated protein kinase (MAPK) signaling, sulfur metabolism, selenocompound metabolism, valine, leucine and isoleucine degradation, beta-alanine metabolism, cell cycle-yeast, meiosis-yeast, and inositol phosphate metabolism pathways (Figure 7D).

3.7 X33 AMOP suppressed the virulence characteristics of *C. albicans*

The virulence factors, including adhesion, secretion of enzymes, and morphology transformation, are necessary for *C. albicans* pathogenicity (Calderone and Fonzi, 2001). Phospholipase activity was indicated by the Pz values, and higher Pz values corresponded to lower phospholipase activities. The control group had Pz value of 0.42 ± 0.02, while the Pz value of the MIC group was 0.49 ± 0.03

TABLE 2 The quality of sequencing.

Samples	Raw reads (million)	Clean reads (million)	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
C_1	43.01	42.64	0.02	98.73	95.55	37.79
C_2	43.84	43.37	0.02	98.54	95.20	38.19
C_3	43.74	43.16	0.02	98.56	95.24	38.17
E_1	43.28	42.69	0.02	98.18	94.49	37.76
E_2	44.49	44.13	0.02	98.52	95.09	36.96
E_3	44.19	43.61	0.02	98.52	95.14	36.82

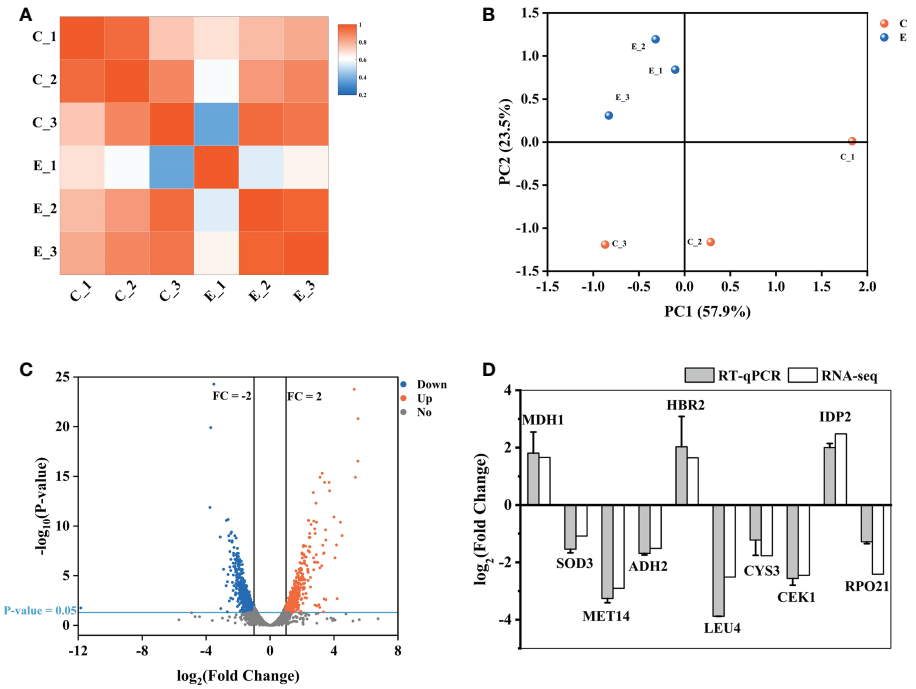

FIGURE 6
Expression analysis of different genes between the samples. **(A)** The biological repeat correlation analysis of the samples. The different colors represent correlation coefficients, and the colors closer to red indicate higher gene expression similarity among the samples. **(B)** Principal component analysis (PCA) of the samples. **(C)** A volcano plot showing the expression analysis of the different genes. The red dots represent significantly up-regulated genes, the blue dots represent significantly down-regulated genes, and the grey dots represent genes without significant expression differences. **(D)** The RT-qPCR results showing the expression of the different genes. The vertical axis represents the fold changes. Up-regulated and down-regulated genes are represented by positive and negative values, respectively.

(Figure 8A), indicating that X33 AMOP could significantly reduce the phospholipase activity. Morphogenesis is a form of growth transformation from yeast and filamentous cells. The yeast cells are more conducive for reproduction, while hyphal (filamentous) ones are more suitable for invasion and virulence (Gong et al., 2019). As shown in Figure 8B, the transformation from yeast to hyphal cells was inhibited by X33 AMOP in a dose-dependent manner. Long and interlaced pseudo hyphae or hyphae were detected in the control

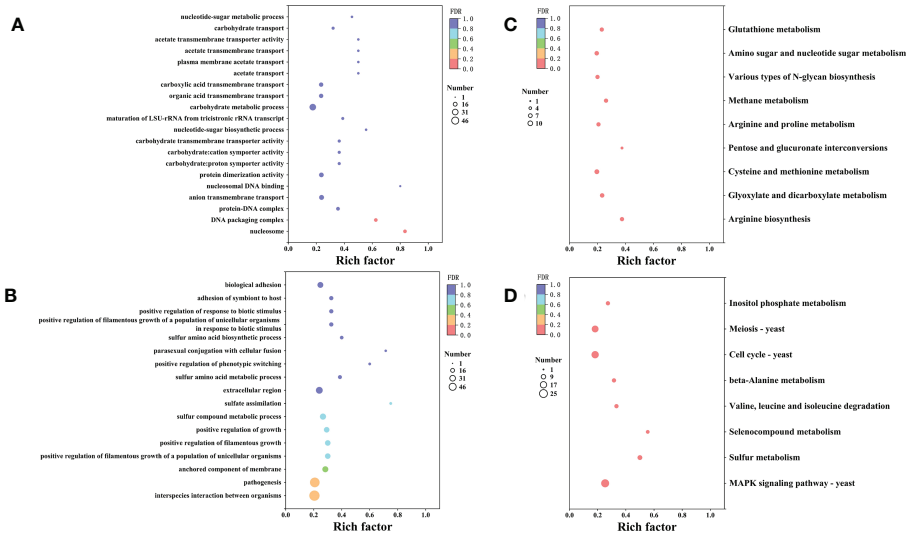

FIGURE 7
The GO and KEGG enrichment analyses of the differentially expressed genes. The vertical axis represents GO Term or KEGG Pathway, while the horizontal axis represents the enrichment factor. The dot size represents the number of genes in the GO Term or KEGG Pathway, and the dot color corresponds to the different P-values. **(A)** The top 20 GO functions of the up-regulated genes. **(B)** The top 20 GO functions of the down-regulated genes. **(C)** The top 20 KEGG pathways enrichment of the up-regulated genes. **(D)** The top 20 KEGG pathways enrichment of the up-regulated genes.

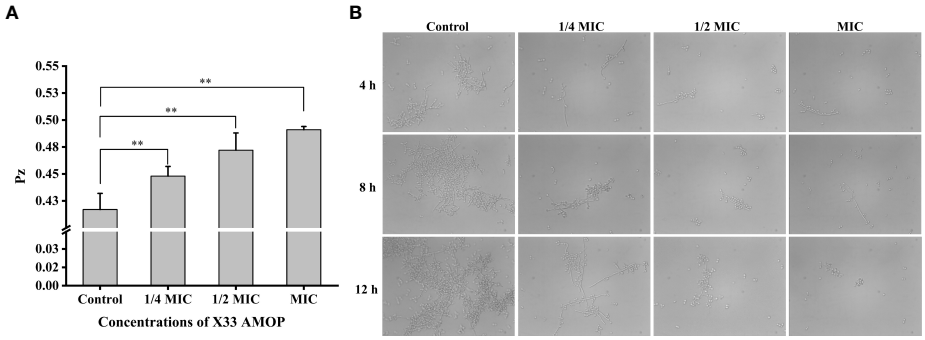

FIGURE 8
Effects of X33 AMOP on virulent attributes of (*C*) *albicans*. **(A)** The phospholipase activity values (Pz values) at the different X33 AMOP concentrations. **(B)** The transformation morphologies of (*C*) *albicans*. *$p < 0.05$, **$p < 0.01$.

group, while the X33 AMOP treatment group displayed single-yeast cells and a few filaments. These results indicated that X33 AMOP treatment could inhibit the virulence of *C. albicans*.

4 Discussions

In the present study, we investigated the inhibitory mechanism of X33 AMOP against *C. albicans* through transcriptome and physiological analysis. The MIC, MFC, and bacteriostatic circle diameter results showed that X33 AMOP inhibited *C. albicans* in a dose-dependent manner. Similarly, the growth curve showed that X33 AMOP significantly inhibited the growth of *C. albicans*. The number of cells, germ tubes, spores, and hypha were significantly decreased, and the *C. albicans* cells treated with X33 AMOP showed shrunken, deformed and irregular morphologies. Further morphological analysis using TEM indicated the *C. albicans* cells treated with X33 AMOP had ruptured cell boundaries, shrunken organelles, and leaked cellular contents. Thus, these phenotypic and physiological analyses illustrated that X33 AMOP has a strong inhibitory effect on *C. albicans*.

4.1 Cell wall integrity of *C. albicans* in response to X33 AMOP

Damage to any cell wall component can trigger various reactions, leading to the compensatory formation of another competent cell wall (Popolo et al., 2001). After the X33 AMOP treatment, the expression of genes, including *CHS1* (gene-CAALFM_C702770WA), *UTR2* (gene-CAALFM_C301730CA), *GSC1* (gene-CAALFM_C102420CA), *PHR2* (gene-CAALFM_C100220WA), and *PMT4* (gene-CAALFM_C206100WA), related to the synthesis of cell wall components was downregulated (Table 3). In contrast, the expression of *CHT2, CRH11, CHS7, GFA1, UAP1, GNA1, XOG1, SGA1*, and *MNN22* were upregulated after the treatment with X33 AMOP (Table 3). Although the chitin synthesis-related genes (*CSH1* and *UTR2*) were downregulated, the cell wall damage triggered the upregulation of cell wall related genes (*UADP1*, GFA1, *CHR11*, and *CSH7*) as compensatory response mechanisms (Lagorce et al., 2002). Interestingly, a previous study showed that when treated with micafungin, the expression of *CHT2*, *CHS1*, and *UTR2* was upregulated (Kaneko et al., 2010), contrary to our findings. The genes encoding 1,3-beta-glucan synthase (*GSC1* and *GSL2*), 1,3-beta-glucanosyltransferase (*PHR2* and *PHR3*), beta-glucan synthesis-associated protein (*ENG1*, *KRE6*, and *BGL22*), and glucan glucosidase (*XOG1*, *SGA1*, and *BGL2*) were mainly involved in beta-glucan biosynthesis. Moreover, these genes showed significant differences in their expression after the X33 AMOP treatment. Similarly, another study showed that after induction by fluconazole, the expression of *XOG1* and *BGL2*, involved in 1,3-beta-glucan transportation, were upregulated (Shao et al., 2017). In this study, we found that the X33 AMOP-induced genes encoding olichyl-phosphate-mannose-protein mannosyltransferase (*PMT1* and *PMT4*) and mannan endo-1,6-alpha-mannosidase (*DFG5*) were significantly downregulated. However, the genes encoding Alpha-1,2-mannosyltransferase (*MNN22*, *MNN23* and *MN24*) and mannan endo-1,6-alpha-mannosidase (*MNN9*) were upregulated. A previous study reported that echinocandins inhibited the synthesis of 1,3-beta-glucan, resulting in a compensatory increase of chitin content in the cell wall, which triggered the expression of mannoprotein for reshaping the cell wall (Ibe et al., 2017).

The extracellular AKP activities in *C. albicans* significantly increased after the X33 AMOP treatment, further confirming the cell wall disruption caused by X33 AMOP. Consistently, the peptide (C12H23O)-OOWW-NH2 (C12O3TR) and cinnamaldehyde reportedly increased the AKP activities and cell wall damage (OuYang et al., 2019; Li et al., 2020). Furthermore, the number of viable cells under X33 AMOP treatment increased with the addition of 0.8 M sorbitol, demonstrating similar effects to that of geraniol and citronellol on the cell wall of *Trichophyton rubrum* (Pereira Fde et al., 2015).

4.2 Cell membrane integrity of *C. albicans* in response to X33 AMOP

Ergosterol is an essential and specific component of the cell membrane responsible for adjusting the fluidity, maintaining integrity, and modulating the transportation process of the cells (Ma et al., 2020). Ergosterol biosynthesis is a complex process involving multiple genes called ERG. We found that *ERG3* (gene-

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TABLE 3 Differentially expression genes in *C. albicans* after X33 AMOP treatment.

Gene ID	Gene name	Gene description	Log2FC(E/C)	Padjust	Regulate
Cell wall synthesis					
gene-CAALFM_C202010CA	CHT4	putative chitinase	-1.64	0.00	down
gene-CAALFM_C504130CA	CHT2	Cht2p	2.30	0.00	up
gene-CAALFM_C502530WA	UAP1	UDP-N-acetylglucosamine diphosphorylase	3.12	0.00	up
gene-CAALFM_C203870WA	GNA1	glucosamine 6-phosphate N-acetyltransferase	1.54	0.01	up
gene-CAALFM_C302280CA	GFA1	glutamine–fructose-6-phosphate transaminase (isomerizing)	2.79	0.00	up
gene-CAALFM_C402900CA	CRH11	transglycosylase	2.06	0.00	up
gene-CAALFM_C702770WA	CHS1	chitin synthase	-1.21	0.01	down
gene-CAALFM_C106010WA	CHS7	Chs7p	1.65	0.00	up
gene-CAALFM_C301730CA	UTR2	Utr2p	-2.28	0.00	down
gene-CAALFM_C102420CA	GSC1	1,3-beta-glucan synthase	-1.44	0.00	down
gene-CAALFM_CR00850CA	GSL2	Gsl2p	-1.35	0.00	down
gene-CAALFM_C100220WA	PHR2	1,3-beta-glucanosyltransferase	-2.73	0.00	down
gene-CAALFM_C400090WA	PHR3	1,3-beta-glucanosyltransferase	-1.33	0.02	down
gene-CAALFM_C103680WA	ENG1	endo-1,3(4)-beta-glucanase	-1.19	0.02	down
gene-CAALFM_C305830WA	KRE6	beta-glucan synthesis-associated protein	-1.21	0.01	down
gene-CAALFM_CR09420CA	BGL22	Bgl22p	-1.20	0.03	down
gene-CAALFM_C102990CA	XOG1	glucan 1,3-beta-glucosidase	2.19	0.00	up
gene-CAALFM_C301320CA	SGA1	glucan 1,4-alpha-glucosidase	1.90	0.00	up
gene-CAALFM_CR09420CA	BGL22	Bgl22p	-1.20	0.03	down
gene-CAALFM_C206100WA	PMT4	dolichyl-phosphate-mannose-protein mannosyltransferase	-1.95	0.00	down
gene-CAALFM_C702890CA	PMT1	dolichyl-phosphate-mannose-protein mannosyltransferase	-1.53	0.01	down
gene-CAALFM_C306020WA	MNN9	mannosyltransferase complex subunit	1.21	0.02	up
gene-CAALFM_C110070CA	MNN23	Mnn23p	1.40	0.02	up
gene-CAALFM_C305610WA	MNN14	Mnn14p	1.42	0.01	up
gene-CAALFM_C201300CA	MNN24	Mnn24p	1.60	0.00	up
gene-CAALFM_C110300WA	MNN12	alpha-1,3-mannosyltransferase	1.68	0.00	up
gene-CAALFM_C404770CA	MNN22	Mnn22p	2.36	0.00	up
gene-CAALFM_C200520WA	DFG5	putative mannan endo-1,6-alpha-mannosidase	-1.03	0.02	down
Cell membrane synthesis					
gene-CAALFM_C300760WA	ERG4	Delta (24(24(1)))-sterol reductase	1.22	0.01	up
gene-CAALFM_C104770CA	ERG3	C-5 sterol desaturase	1.32	0.00	up
gene-CAALFM_C108460CA	UPC2	Upc2p	-1.90	0.00	down
gene-CAALFM_C504910WA	CAALFM_C504910WA	hypothetical protein	-1.78	0.00	down
Oxidative stress					
gene-CAALFM_C200680CA	SOD5	Sod5p	-2.34	0.00	down
gene-CAALFM_C700110WA	SOD3	Sod3p	-1.08	0.03	down
energy metabolism					
gene-CAALFM_C300880WA	KGD1	alpha-ketoglutarate dehydrogenase	-1.84	0.00	down
gene-CAALFM_C403940CA	PYC2	pyruvate carboxylase 2	-1.51	0.00	down

(Continued)

TABLE 3 Continued

Gene ID	Gene name	Gene description	Log2FC(E/C)	Padjust	Regulate
gene-CAALFM_C701640WA	LAT1	dihydrolipoyllysine-residue acetyltransferase	-1.18	0.03	down
gene-CAALFM_C105260CA	SDH1	succinate dehydrogenase flavoprotein subunit	-1.08	0.04	down
gene-CAALFM_C401850CA	PDC12	Pdc12p	-1.19	0.04	down
gene-CAALFM_C100170WA	LEU4	2-isopropylmalate synthase	-2.51	0.00	down
gene-CAALFM_CR04530WA	FUM11	fumarase	1.03	0.01	up
gene-CAALFM_CR00540CA	MDH1	malate dehydrogenase	1.66	0.00	up
gene-CAALFM_CR02360WA	IDP2	isocitrate dehydrogenase (NADP(+))	2.48	0.00	up
gene-CAALFM_C101390CA	CAALFM_C101390CA	hypothetical protein	-1.92	0.00	down
gene-CAALFM_C404010WA	CAALFM_C404010WA	hypothetical protein	-1.08	0.00	down
gene-CAALFM_C104320WA	GPM2	Gpm2p	1.32	0.00	up
Virulence factor					
gene-CAALFM_C206170CA	ECM17	sulfite reductase (NADPH) subunit beta	-2.55	0.00	down
gene-CAALFM_C104020CA	CSH1	Csh1p	-2.04	0.00	down
gene-CAALFM_C203040WA	PLC2	Plc2p	-1.80	0.00	down
gene-CAALFM_C111590WA	PLD1	phospholipase D	-1.40	0.01	down
gene-CAALFM_C406480CA	CEK1	mitogen-activated serine/threonine-protein kinase	-2.45	0.00	down
gene-CAALFM_CR05940WA	CEK2	Cek2p	-1.96	0.00	down
gene-CAALFM_C302100WA	STE50	Ste50p	-1.63	0.00	down
gene-CAALFM_C502340CA	CST20	mitogen-activated protein kinase kinase kinase kinase	-1.33	0.00	down
gene-CAALFM_CR03900WA	HST7	mitogen-activated protein kinase kinase	-1.11	0.05	down

CAALFM_C104770CA) and *ERG4* (gene-CAALFM_C300760WA) were upregulated in *C. albicans* after the X33 AMOP treatment. Overexpression of *ERG3*, encoding $\Delta^{5,6}$-desaturase, enhanced the synthesis of toxic steroids, casing cell damage (Sanglard et al., 2003; Prasad et al., 2019). However, upregulating *ERG4*, encoding delta (24 (24(1)))-sterol reductase, which catalyzes the conversion of 5,7,22,24 (28)-ergostate traenol to ergosterol, increased the ergosterol biosynthesis (Feng et al., 2017). These results indicate that X33 AMOP could interfere with the biosynthesis of cell membrane components, thus destroying its integrity. The fluorescence microscope scan, scanning electron microscope, and the increase of MDA contents also suggested that the biosynthesis and integrity of the *C. albicans* membrane were damaged by X33 AMOP. Therefore, X33 AMOP exerts inhibitory effects on protein biosynthesis and carbon metabolism by damaging the cell membrane, causing the leakage of cell contents in *C. albicans*.

4.3 Oxidative damage of *C. albicans* in response to X33 AMOP

The accumulation of ROS in cell mitochondria destroys various cellular components, such as protein and lipids, thus disrupting the synthesis of ATP (Wellen and Thompson, 2010). Simultaneously, cellular accumulation of ROS leads to membrane phospholipid peroxidation, which generates MDA (Su et al., 2020). In the present study, ROS significantly increased in the cells treated with X33 AMOP, suggesting that oxidative stress caused by ROS production exacerbated the cell damage of *C. albicans*. This also resulted in imbalanced redox system and increased MDA contents, indicating that X33 AMOP treatment also caused mitochondrial damage. The general mechanism of antifungal drugs against pathogenic fungi involves the accumulation of hydrogen peroxide (Delattin et al., 2014). Therefore, superoxide dismutase, which converts superoxide radicals into hydrogen peroxide, was introduced into *C. albicans* to reduce oxidative stress (Broxton and Culotta, 2016). In the present study, the expression of *SOD3* (gene-CAALFM_C700110WA), encoding iron/manganese superoxide dismutases, and *SOD5* (gene-CAALFM_C200680CA), encoding copper/zinc superoxide dismutase, were downregulated in *C. albicans* after the X33 AMOP treatment. Consistently, the expression of Cu/Zn superoxide dismutase was also downregulated in *Procambarus clarkii* under ammonia stress (Wang et al., 2020). Exposure to high H_2O_2 levels induced by X33 AMOP caused the cell death of *C. albicans*, indicating that X33 AMOP can cause oxidative stress in *C. albicans* and further induce cell death. Similarly, increased ROS and lipid peroxidation and

upregulated expression of SODs was observed in *C. albicans* treated with honokiol (Sun et al., 2017).

4.4 Energy synthesis changes in *C. albicans* in response to X33 AMOP

Pyruvate is an important intermediate metabolite generated during the transformation of sugar, fatty acid, and amino acids through acetyl-CoA and TCA cycles. We found that various crucial genes, including *KGD1* (gene-CAALFM_C300880WA), *PYC2* (gene-CAALFM_C403940CA), *LEU4* (gene-CAALFM_C100170WA), and *CAALFM_C101390CA* (gene-CAALFM_C101390CA), involved in pyruvate metabolism, the glycolytic pathway, and TCA cycle were downregulated. However, *MDH1* (gene-CAALFM_CR00540CA), *IDP2* (gene-CAALFM_CR02360WA), and *GPM2* (gene-CAALFM_C104320WA) were upregulated in *C. albicans* in response to X33 AMOP. The glycolytic pathway is the central metabolic pathway for carbon metabolism, which converts glucose to pyruvate and simultaneously generates ATP and NADH. The domain analysis of the hypothetical protein (encoded by *gene-CAALFM_C101390CA*) indicated its role as a transcriptional activator of glycolytic enzymes, predominantly responsible for activating the expression of glycolytic genes, which regulate the pathway (Sasaki et al., 2005). Although the expression of *GPM2*, encoding the enzyme which converts 3-phosphoglycerate to 2-phosphoglycerate, was upregulated, the transcriptional activator and the genes involved in diverting fructose 6-phosphate to the chitin synthesis pathway were downregulated. This was due to the cell wall compensation mechanism and the negative effects exerted on pyruvate biosynthesis. Several genes involved in the TCA cycle were significantly downregulated, indicating disruption of energy metabolism in *C. albicans*. Among them, *PYC2* encodes pyruvate carboxylase 2, which catalyzes the conversion of pyruvate to oxaloacetic acid, while *KGD1* encodes alpha-ketoglutarate dehydrogenase catalyzing the conversion of alpha-ketoglutarate to succinyl coenzyme A and NADP. Furthermore, the downregulation of *PYC2* and *PDC12* reduced the metabolic flux of the TCA cycle, consistent with the downregulation of HSAF in the TCA cycle and the expression glycolysis genes in *Alternaria alternate* (He et al., 2018). Downregulation of *LEU4*, encoding 2-isopropylmalate synthetase, affected pyruvate synthesis by inhibiting the conversion of 2-isopropylmalate from 2-ketoisovalerate. Furthermore, the physiological parameter analysis showed that the pyruvic acid content decreased after the X33 AMOP treatment and that the gene expression changes were consistent with the phenotype characteristics. The scanning electron microscopy showed that the organelles of the *C. albicans* treated with X33 AMOP were clustered, indicating that their functions were damaged. The intracellular pH stability is tightly controlled by the plasma membrane and vacuolar H+-ATPases (Tsang et al., 2014). Thus, the inhibition of proton extrusion greatly decreases the pH. In a present study, the extracellular medium acidification of the X33 treatment group was lower than that of the control group, indicating that the ATPase activities induced by X33 AMOP mediated the proton efflux activity of *C. albicans*. This is consistent with a previous finding showing that the pH of *C. albicans* treated with cinnamic aldehydes was higher than that of the control group (Shreaz et al., 2013). The cell wall components, soluble protein, and reducing sugar are important for cellular activities (Chen et al., 2020a). Similar to pyruvate, the protein and soluble sugar contents decreased in *C. albicans* cells treated with X33 AMOP. This demonstrates that X33 AMOP interfered with the expression of genes involved in glycolysis, TCA cycle and pyruvate metabolism, consequently hindering energy metabolism and altering the mitochondrial function of *C. albicans* cells.

4.5 Virulence change in *C. albicans* in response to X33 AMOP

Attenuating the virulence factors is the main inhibitory effect exhibited by antifungal drugs (Talapko et al., 2021). In this study, we found that the morphological transformation and phospholipase activity of *C. albicans* were inhibited after the X33 AMOP treatment. The expression of the hyphal formation and virulence-related genes was downregulated by approximately 1.40- to 2.45-fold. These gene included *ECM17* (gene-CAALFM_C206170CA), *CEK1* (gene-CAALFM_C406480CA), *CEK2* (gene-CAALFM_CR05940WA), *PLC2* (gene-CAALFM_C203040WA), *PLD1* (gene-CAALFM_C111590WA), and *STE50* (gene-CAALFM_C302100WA). The biosynthesis of methionine and cysteine is regulated by *ECM17*. The deficiency of these amino acids affects the expression of various proteins, disrupts the activities of signaling pathways, and interferes with the adhesion and filamentous growth in *C. albicans* (Li et al., 2013). *CEK1* and *CEK2*, encoding the extracellular signal-regulated kinase (CEK), were involved in the MAPK pathway, mainly responsible for the hyphal mating, morphological transformation, and cell wall stress adaptation in *C.albicans* (Chen et al., 2020b). Moreover, deleting the *CEK1*, *CST20*, and *HST7*, inhibited hyphal morphogenesis in *C. albicans* (Leberer et al., 1996; Csank et al., 1998). It is speculated that downregulating these genes may also affect hyphal formation. *PLD1* exhibits a high-level expression when *C. albicans* changes from yeast to hyphal growth form, probably due to the biosynthesis of phosphatidic acid and diacylglycerol (Hube et al., 2001; Dolan et al., 2004).

5 Conclusion

This study reveals the detailed molecular mechanisms of X33 AMOP against *C. albicans* through transcriptomic analysis and changes in physiological parameters. Our results show that X33 AMOP affects the cellular components and damages the integrity of the membranes and cell walls by stimulating oxidative stress and inducing mitochondrial dysfunction in *C. albicans*. This shows that X33 AMOP exhibits significant inhibitory effects on the virulence of *C. albicans*. Thus, this study provides possible targets for X33 AMOP against *C. albicans* and a theoretical basis for the antifungal activity of antimicrobial peptides.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

QL conducted the experiments, wrote the first draft, and revised the draft. provided investigation and experimental technique support, and performed the response of the draft. BZ and XW designed the experiments, provided guidance for the experiments, prepared the manuscript, and provided financial support for the experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1123393/full#supplementary-material

References

Antinori, S., Milazzo, L., Sollima, S., Galli, M., and Corbellino, M. (2016). Candidemia and invasive candidiasis in adults: a narrative review. *Eur. J. Intern. Med.* 34, 21–28. doi: 10.1016/j.ejim.2016.06.029

Berdy, J. (2012). Thoughts and facts about antibiotics: where we are now and where we are heading. *J. Antibio.* 65 (8), 385–395. doi: 10.1038/ja.2012.27

Broxton, C. N., and Culotta, V. C. (2016). SOD enzymes and microbial pathogens: surviving the oxidative storm of infection. *PloS Pathog.* 12 (1), e1005295. doi: 10.1371/journal.ppat.1005295

Calderone, R. A., and Fonzi, W. A. (2001). Virulence factors of *Candida albicans*. *Trend. Microbiol.* 9 (7), 327–335. doi: 10.1016/s0966-842x(01)02094-7

Chen, C.-J., Li, Q.-Q., Zeng, Z.-Y., Duan, S.-S., Wang, W., Xu, F.-R., et al. (2020a). Efficacy and mechanism of *Mentha haplocalyx* and *Schizonepeta tenuifolia* essential oils on the inhibition of *Panax notoginseng* pathogens. *Ind. Crop Prod.* 145, 112073. doi: 10.1016/j.indcrop.2019.112073

Chen, H., Zhou, X., Ren, B., and Cheng, L. (2020b). The regulation of hyphae growth in *Candida albicans*. *Virulence* 11 (1), 337–348. doi: 10.1080/21505594.2020.1748930

Csank, C., Schroppel, K., Leberer, E., Harcus, D., Mohamed, O., Meloche, S., et al. (1998). Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infect. Immun.* 66 (6), 2713–2721. doi: 10.1128/IAI.66.6.2713-2721.1998

da Silva Neto, J. X., da Costa, H. P. S., Vasconcelos, I. M., Pereira, M. L., Oliveira, J. T. A., Lopes, T. D. P., et al. (2020). Role of membrane sterol and redox system in the anti-candida activity reported for *Mo*-CBP2, a protein from *Moringa oleifera* seeds. *Int. J. Biol. Macromol.* 143, 814–824. doi: 10.1016/j.ijbiomac.2019.09.142

Delattin, N., Cammue, B. P., and Thevissen, K. (2014). Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. *Future Med. Chem.* 6 (1), 77–90. doi: 10.4155/fmc.13.189

Dimopoulos, G., Karabinis, A., Samonis, G., and Falagas, M. E. (2007). Candidemia in immunocompromised and immunocompetent critically ill patients: a prospective comparative study. *Eur. J. Clin. Microbiol. Infect. Dis.* 26 (6), 377–384. doi: 10.1007/s10096-007-0316-2

Dolan, J. W., Bell, A. C., Hube, B., Schaller, M., Warner, T. F., and Balish, E. (2004). *Candida albicans* PLDI activity is required for full virulence. *Med. Mycol.* 42 (5), 439–447. doi: 10.1080/13693780410001657162

Feng, W., Yang, J., Xi, Z., Qiao, Z., Lv, Y., Wang, Y., et al. (2017). Mutations and/or overexpressions of *ERG4* and *ERG11* genes in clinical azoles-resistant isolates of *Candida albicans*. *Microb. Drug Resist.* 23 (5), 563–570. doi: 10.1089/mdr.2016.0095

Fu, J., Ding, Y., Wei, B., Wang, L., Xu, S., Qin, P., et al. (2017). Epidemiology of *Candida albicans* and non-*C. albicans* of neonatal candidemia at a tertiary care hospital in western China. *BMC Infect. Dis.* 17 (1), 329. doi: 10.1186/s12879-017-2423-8

Ghannoum, M. A., Jurevic, R. J., Mukherjee, P. K., Cui, F., Sikaroodi, M., Naqvi, A., et al. (2010). Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PloS Pathog.* 6 (1), e1000713. doi: 10.1371/journal.ppat.1000713

Gong, Y., Liu, W., Huang, X., Hao, L., Li, Y., and Sun, S. (2019). Antifungal activity and potential mechanism of n-butylphthalide alone and in combination with fluconazole against *Candida albicans*. *Front. Microbiol.* 101461. doi: 10.3389/fmicb.2019.01461

He, F., Li, B., Ai, G., Kange, A. M., Zhao, Y., Zhang, X., et al. (2018). Transcriptomics analysis of the chinese pear pathotype of *Alternaria alternata* gives insights into novel mechanisms of HSAF antifungal activities. *Int. J. Mol. Sci.* 19 (7), 1841. doi: 10.3390/ijms19071841

Hoehamer, C. F., Cummings, E. D., Hilliard, G. M., and Rogers, P. D. (2010). Changes in the proteome of *Candida albicans* in response to azole, polyene, and echinocandin antifungal agents. *Antimicrob. Agents Chemother.* 54 (5), 1655–1664. doi: 10.1128/AAC.00756-09

Huan, Y., Kong, Q., Mou, H., and Yi, H. (2020). Antimicrobial peptides: Classification, design, application and research progress in multiple fields. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.582779

Hube, B., Hess, D., Baker, C. A., Schaller, M., Schafer, W., and Dolan, J. W. (2001). The role and relevance of phospholipase D1 during growth and dimorphism of *Candida albicans*. *Microbiol. (Reading).* 147 (Pt 4), 879–889. doi: 10.1099/00221287-147-4-879

Ibe, C., Walker, L. A., Gow, N. A. R., and Munro, C. A. (2017). "Unlocking the therapeutic potential of the fungal cell wall: clinical implications and drug resistance," in *Candida albicans: Cellular and molecular biology*. Ed. R. Prasad (Cham: Springer). doi: 10.1007/978-3-319-50409-4_16

Kaneko, Y., Ohno, H., Kohno, S., and Miyazaki, Y. (2010). Micafungin alters the expression of genes related to cell wall integrity in *Candida albicans* biofilms. *Jpn. J. Infect. Dis.* 63 (5), 355–357. doi: 10.7883/yoken.63.355

Lagorce, A., Le Berre-Anton, V., Aguilar-Uscanga, B., Martin-Yken, H., Dagkessamanskaia, A., and Francois, J. (2002). Involvement of *GFA1*, which encodes glutamine-fructose-6-phosphate amidotransferase, in the activation of the chitin synthesis pathway in response to cell-wall defects in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 269 (6), 1697–1707. doi: 10.1046/j.1432-1327.2002.02814.x

Leberer, E., Harcus, D., Broadbent, I. D., Clark, K. L., Dignard, D., Ziegelbauer, K., et al. (1996). Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. *Proc. Natl. Acad. Sci. U.S.A.* 93 (23), 13217–13222. doi: 10.1073/pnas.93.23.13217

Leite, M. C., Bezerra, A. P., de Sousa, J. P., Guerra, F. Q., and Lima Ede, O. (2014). Evaluation of antifungal activity and mechanism of action of citral against *Candida albicans*. *Evid. Based Complement. Alternat. Med.* 2014, 378280. doi: 10.1155/2014/378280

Li, X., Feng, G., Wang, W., Yi, L., Deng, L., and Zeng, K. (2020). Effects of peptide C12-OOWW-NH2 on transcriptome and cell wall of the postharvest fungal pathogen *Penicillium digitatum*. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.574882

Lin, S. H., Luo, P., Yuan, E., Zhu, X., Zhang, B., and Wu, X. (2020). Physiological and proteomic analysis of penicillium digitatum in response to X33 antifungal extract treatment. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.584331

Li, D. D., Wang, Y., Dai, B. D., Li, X. X., Zhao, L. X., Cao, Y. B., et al. (2013). *ECM17*-dependent methionine/cysteine biosynthesis contributes to biofilm formation in *Candida albicans*. *Fungal Genet. Biol.* 51, 50–59. doi: 10.1016/j.fgb.2012.11.010

Ma, H., Zhao, X., Yang, L., Su, P., Fu, P., Peng, J., et al. (2020). Antimicrobial peptide AMP-17 affects *Candida albicans* by disrupting its cell wall and cell membrane integrity. *Infect. Drug Resist.* 13, 2509–2520. doi: 10.2147/IDR.S250278

OuYang, Q., Duan, X., Li, L., and Tao, N. (2019). Cinnamaldehyde exerts its antifungal activity by disrupting the cell wall integrity of *Geotrichum citri-aurantii*. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.00055

Pappas, P. G., Kauffman, C. A., Andes, D. R., Clancy, C. J., Marr, K. A., Ostrosky-Zeichner, L., et al. (2016). Clinical practice guideline for the management of candidiasis: 2016 update by the infectious diseases society of america. *Clin. Infect. Dis.* 62 (4), e1–50. doi: 10.1093/cid/civ933

Patriota, L. L., Procopio, T. F., de Souza, M. F., de Oliveira, A. P., Carvalho, L. V., Pitta, M. G., et al. (2016). A trypsin inhibitor from *Tecoma stans* leavesinhibits growth and promotes ATP depletion and lipid peroxidation in *Candida albicans* and *Candida krusei*. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.00611

Pereira Fde, O., Mendes, J. M., Lima, I. O., Mota, K. S., Oliveira, W. A., and Lima Ede, O. (2015). Antifungal activity of geraniol and citronellol, two monoterpenes alcohols, against *Trichophyton rubrum* involves inhibition of ergosterol biosynthesis. *Pharm. Biol.* 53 (2), 228–234. doi: 10.3109/13880209.2014.913299

Popolo, L., Gualtieri, T., and Ragni, E. (2001). The yeast cell-wall salvage pathway. *Med. Mycol.* 39 (1), 111–121. doi: 10.1080/714031004

Prasad, R., Nair, R., and Banerjee, A. (2019). Emerging mechanisms of drug resistance in *Candida albicans*. *Prog. Mol. Subcell. Biol.* 58, 135–153. doi: 10.1007/978-3-030-13035-0_6

Priya, A., and Pandian, S. K. (2020). Piperine impedes biofilm formation and hyphal morphogenesis of *Candida albicans*. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.00756

Rauseo, A. M., Coler-Reilly, A., Larson, L., and Spec, A. (2020). Hope on the horizon: novel fungal treatments in development. *Open Forum Infect. Dis.* 7 (2), ofaa016. doi: 10.1093/ofid/ofaa016

Ren, T., Zhu, H., Tian, L., Yu, Q., and Li, M. (2020). *Candida albicans* infection disturbs the redox homeostasis system and induces reactive oxygen species accumulation for epithelial cell death. *FEMS Yeast. Res.* 20 (4), foz081. doi: 10.1093/femsyr/foz081

Ross, D. D., Joneckis, C. C., Ordonez, J. V., Sisk, A. M., Wu, R. K., Hamburger, A. W. N. R. E., et al. (1989). Estimation of cell survival by flow cytometric quantification of fluorescein diacetate/propidium iodide viable cell number. *Cancer Res.* 49 (14), 3776–3782. doi: 10.1016/0304-3835(89)90024-4

Ruhnke, M. (2006). Epidemiology of *Candida albicans* infections and role of non-*Candida-albicans* yeasts. *Curr. Drug Targets* 7 (4), 495–504. doi: 10.2174/138945006776359421

Sanglard, D., Ischer, F., Parkinson, T., Falconer, D., and Bille, J. (2003). *Candida albicans* mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. *Antimicrob. Agents Chemother.* 47 (8), 2404–2412. doi: 10.1128/AAC.47.8.2404-2412.2003

Sasaki, H., Kishimoto, T., Mizuno, T., Shinzato, T., and Uemura, H. (2005). Expression of *GCR1*, the transcriptional activator of glycolytic enzyme genes in the yeast *Saccharomyces cerevisiae*, is positively autoregulated by Gcr1p. *Yeast* 22 (4), 305–319. doi: 10.1002/yea.1212

Shao, J., Cui, Y., Zhang, M., Wang, T., Wu, D., and Wang, C. (2017). Synergistic *in vitro* activity of sodium houttuyfonate with fluconazole against clinical *Candida albicans* strains under planktonic growing conditions. *Pharm. Biol.* 55 (1), 355–359. doi: 10.1080/13880209.2016.1237977

Shi, C., He, Y., Feng, X., and Fu, D. (2015). ϵ-polylysine and next-generation dendrigraft poly-l-lysine: chemistry, activity, and applications in biopharmaceuticals. *J. Biomater. Sci. Polym. Ed.* 26 (18), 1343–1356. doi: 10.1080/09205063.2015.1095023

Shreaz, S., Bhatia, R., Khan, N., Muralidhar, S., Manzoor, N., and Khan, L. A. (2013). Influences of cinnamic aldehydes on plasma membrane h^+ ATPase activity and ultrastructure of *Candida*. *J. Med. Microbiol.* 62 (Pt 2), 232–240. doi: 10.1099/jmm.0.036145-0

Sivalingam, P., Hong, K., Pote, J., and Prabakar, K. (2019). Extreme environment *Streptomyces*: Potential sources for new antibacterial and anticancer drug leads? *Int. J. Microbiol.* 2019, 5283948. doi: 10.1155/2019/5283948

Song, M., Wang, X., Mao, C., and Yao, W. (2018). The discovery of a potential antimicrobial agent: the novel compound natural medicinal plant fermentation extracts against *Candida albicans*. *IOP. Conf. Series.: Materials. Sci. Eng.* 301. doi: 10.1088/1757-899x/301/1/012026

Srivastava, V., Singla, R. K., and Dubey, A. K. (2018). Inhibition of biofilm and virulence factors of *Candida albicans* by partially purified secondary metabolites of *Streptomyces chrestomyceticus* strain ADP4. *Curr. Top. Med. Chem.* 18 (11), 925–945. doi: 10.2174/1568026618666180711154110

Su, H., Hu, C., Cao, B., Qu, X., Guan, P., Mu, Y., et al. (2020). A semisynthetic borrelidin analogue BN-3b exerts potent antifungal activity against *Candida albicans* through ROS-mediated oxidative damage. *Sci. Rep.* 10 (1), 5081. doi: 10.1038/s41598-020-61681-0

Sun, L., Liao, K., Hang, C., and Wang, D. (2017). Honokiol induces reactive oxygen species-mediated apoptosis in *Candida albicans* through mitochondrial dysfunction. *PloS One* 12 (2), e0172228. doi: 10.1371/journal.pone.0172228

Sun, C. Q., Peng, J., Yang, L. B., Jiao, Z. L., Zhou, L. X., Tao, R. Y., et al. (2022). A cecropin-4 derived peptide C18 inhibits *Candida albicans* by disturbing mitochondrial function. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.872322

Svetaz, L., Aguero, M. B., Alvarez, S., Luna, L., Feresin, G., Derita, M., et al. (2007). Antifungal activity of *Zuccagnia punctata* cav.: evidence for the mechanism of action. *Planta. Med.* 73 (10), 1074–1080. doi: 10.1055/s-2007-981561

Talapko, J., Juzbasic, M., Matijevic, T., Pustijanac, E., Bekic, S., Kotris, I., et al. (2021). *Candida albicans*-the virulence factors and clinical manifestations of infection. *J. Fungi. (Basel).* 7 (2), 79. doi: 10.3390/jof7020079

Tsang, P. W., Wong, A. P., Jung, H. S., and Fong, W. P. (2014). Sub-MIC levels of purpurin inhibit membrane ATPase-mediated proton efflux activity in the human fungal pathogen *Candida albicans*. *J. Antibio.* 67 (4), 349–350. doi: 10.1038/ja.2013.140

Wang, L., Wang, H., Shi, W., Zhang, Y., Chang, G., Wu, N., et al. (2020). RNA-Seq analysis uncovers effects of ammonia on metabolism, oxidant-antioxidant equilibrium and apoptosis in the red swamp crayfish (*Procambarus clarkii*). *Aquacult. Rep.* 18 (2020), 100459. doi: 10.1016/j.aqrep.2020.100459

Wang, T., Xiu, J., Zhang, Y., Wu, J., Ma, X., Wang, Y., et al. (2017). Transcriptional responses of *Candida albicans* to antimicrobial peptide MAF-1A. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00894

Wellen, K. E., and Thompson, C. B. (2010). Cellular metabolic stress: considering how cells respond to nutrient excess. *Mol. Cell* 40 (2), 323–332. doi: 10.1016/j.molcel.2010.10.004

Zou, L., Hu, Y. Y., and Chen, W. X. (2015). Antibacterial mechanism and activities of black pepper chloroform extract. *J. Food Sci. Technol.* 52 (12), 8196–8203. doi: 10.1007/s13197-015-1914-0

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Huaxi Yi,
Ocean University of China, China
Qixiao Zhai,
Jiangnan University, China
Faizan Ahmed Sadiq,
Institute for Agricultural, Fisheries and Food Research (ILVO), Belgium

*CORRESPONDENCE
Zhihong Sun
sunzhihong78@163.com

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Postbiotic gel relieves clinical symptoms of bacterial vaginitis by regulating the vaginal microbiota

Xin Shen[1,2,3], Lin Xu[4], Zhiquan Zhang[5], Yitong Yang[5], Pengxian Li[5], Teng Ma[1,2,3], Shuai Guo[1,2,3], Lai-Yu Kwok[1,2,3] and Zhihong Sun[1,2,3]*

[1]Inner Mongolia Key Laboratory of Dairy Biotechnology and Engineering, Inner Mongolia Agricultural University, Hohhot, Inner Mongolia, China, [2]Key Laboratory of Dairy Products Processing, Ministry of Agriculture and Rural Affairs, Inner Mongolia Agricultural University, Hohhot, Inner Mongolia, China, [3]Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, Inner Mongolia, China, [4]Department of Gynecology, Kunming Tongren Hospital, Kunming, Yunnan, China, [5]Qingyitang Industrial Co., Ltd., Yunnan, China

Vaginitis is the most common disease in gynecology. Vaginal dysbiosis is a main reason of bacteria vaginitis (BV), as the disrupted microecological environment facilitates the growth of various vaginal pathogens. The most dominant bacteria in the vaginal microbiota are lactic acid bacteria, which are important for maintaining vaginal health. At present, antibiotics and other drugs are often used in clinical treatment, but there are many adverse reactions and easy to relapse, and the intervention of probiotics can help restore vaginal microbiota and alleviate BV. This study is a human clinical trial of 50 patients with bacterial vaginitis (BV). The alleviation effect of applying a postbiotic gel for one week in BV was evaluated. Changes in patients' clinical indicators of BV (properties of vaginal secretion) and the vaginal microbiota after using the postbiotic gel were monitored. Our results showed that apply the postbiotic gel improved the symptoms of BV, indicated by improvement in the abnormalities of patients' vaginal secretions. After applying the gel, the relative abundance of vaginal lactobacilli increased compared to baseline. Significant negative correlations were found between lactobacilli and potential vaginal pathogens (including *Gardnerella*, *Prevotella*, and *Atopobium*), as well as the abnormalities of the vaginal secretion. Overall, our results showed that applying the postbiotic gel ameliorated BV, and the symptom improvement was accompanied by significant changes in the bacterial vaginal microbiota. Our study provides valuable clinical data in managing BV.

KEYWORDS

bacterial vaginitis, lactobacilli, *Gardnerella*, vaginal microbiota, postbiotic

1 Introduction

Vaginitis is one of the most common infectious diseases in female gynecology. It is mainly due to inflammation or infection of the vaginal area, and the clinical symptoms of which include vaginal itching, irritation, and discharge of secretion with unpleasant odor (Walter, 2022). The incidence rate of vaginitis shows an increasing trend due to changes in

women's lifestyle and living habits, such as the use of vaginal lavage and antibiotic application. The vaginal microbiota is known to play an important role in maintaining the health state and homeostasis in the vagina and prevents from vaginitis. Vaginitis is largely related to local infections by pathogens, which is clinically classified into trichomoniasis, mycotic vaginitis, bacterial vaginitis, and vulvovaginal candidiasis (Xiao et al., 2022). Changes in the pH in the vaginal environment are associated with the development of many gynecological diseases. In healthy women, the vaginal environment is weakly acidic, which selects for a specific spectrum of vaginal resident microorganisms, e.g., *Lactobacillus*, that inhibit the growth of some pathogenic bacteria (Onderdonk et al., 2016). However, changes in the pH in the vaginal environment may cause vaginal dysbiosis, and some (opportunistic) pathogens may consume glycogen and inhibit *Lactobacillus*, neutralizing or even alkalizing the vaginal environment and thus favoring the growth of pathogenic bacteria and gynecological disease development.

The structure of the vaginal microbiota comprises mainly lactobacilli, and this group of microbes is thus indicative of vaginal health and homeostatic for the local acidic environment (pH < 4.5) (Oakley et al., 2008; Zhang et al., 2018; France et al., 2022a). Lactic acid is a major metabolite of lactic acid bacteria that is responsible for maintaining the vaginal pH environment. A high content of lactic acid in the vagina enhances the integrity of the epithelial cell barrier, thereby preventing pathogen invasion (Delgado-Diaz et al., 2022). At present, metronidazole and clindamycin are commonly used in treating vaginitis, but antibiotic use may cause various adverse reactions, such as a gradual increase in bacterial drug resistance and a high recurrence rate (Happel et al., 2020). Interestingly, a previous study found that the mechanism of metronidazole for treating BV relies on regulating the vaginal bacterial microbiota (Armstrong et al., 2022); therefore, the vaginal bacterial microbiota could be considered a therapeutic target for improving BV.

Probiotics are active microorganisms that confer beneficial effects on the host (Sun et al., 2022). A growing body of research suggests that probiotics are a safe and effective treatment, especially in gastrointestinal disorders, metabolic disorders, and vaginal inflammatory conditions (Rostok et al., 2019; Schellekens et al., 2021; Wallace et al., 2022). In BV, probiotics can regulate the balance of female vaginal microbiota by promoting the growth of beneficial bacteria while inhibiting the harmful ones. A randomized double-blind controlled trial of an 11-week intervention with a metronidazole vaginal gel with *Lactobacillus crispatus* CTV-05 (Lactin-V) significantly reduced the recurrence rate of BV (Cohen et al., 2020). Another randomized double-blind study of healthy women found that applying a *Lactobacillus*/lactoferrin formulation product could improve the vaginitis symptoms without adverse events (Russo et al., 2018). A recent study transplanted vaginal microbiota from healthy women and applied probiotics in combination could improve the clinical symptoms of BV and restore a healthy vaginal microbiota (Chen et al., 2021). Furthermore, the application of *Limosilactobacillus fermentum* LF15 and *Lactiplantibacillus plantarum* LP01 ameliorates the symptoms of BV, and these exogenously administered probiotics probably integrated into the host vaginal microbiota and adhered to the epithelial cells of the vaginal mucosa, thus establishing physiological protection (Vicariotto et al., 2014). It was found that the inactivated probiotics also had probiotic effects. In 2021, ISAPP issued a consensus statement of postbiotic, which for the first time provided the official concept of postbiotic, that is, postbiotic is a preparation of inanimate microorganisms and/or its components that is beneficial to the health of the host (Salminen et al., 2021). However, at present, there are few reports about postbiotic in vaginitis, and the role of postbiotic in vaginitis is worthy of further exploration.

Clinical identification of BV mainly relies on traditional methods of pap smear and biochemical analysis (Nugent et al., 1991) instead of direct analysis of the vaginal microbiota. However, since most of the vaginal microbiota are anaerobic bacteria, and their nutritional requirements are demanding, traditional culture and isolation identification methods would not be able to provide a full landscape of the vaginal microbiota. Therefore, it would be of interest to apply next-generation technology in identifying and monitoring changes in the vaginal microbiota in relation to symptom improvement. At present, high-throughput sequencing technology has been widely used in analyzing the microbiota in various environments, including food (Wang et al., 2020) and buccal samples (Chen et al., 2019). High-throughput sequencing technology can analyze the composition of vaginal microbiota as a whole, which is an effective approach supplementary to existing biochemical identification methods.

This study recruited 50 patients with BV and investigated the therapeutic effect of one-week intervention with postbiotic gel. Changes in the severity of leucorrhea, biochemical properties and bacterial microbiota of subjects' vaginal discharge after the gel intervention were analyzed. This study supports that the application of postbiotic gel could improve BV.

2 Materials and methods

2.1 Trial design and volunteer recruitment

This was a clinical trial conducted between January 2020 to March 2020 at the Department of Obstetrics and Gynecology of Kunming Tongren Hospital. Patients with BV were selected for a one-week intervention of a postbiotic gel to measure changes in the properties of vaginal discharge and vaginal microbiota before (day 0) and after (day 7) intervention. All (n = 50) patients diagnosed with BV at this hospital were considered for participation in the study and were screened by hospital professionals for eligibility. The inclusion criteria were: (1) women clinically diagnosed with diagnostic criteria for BV, aged 18-55 years; (2) body mass index between 19-24; (3) no history of heart, liver, lung, kidney, digestive tract, nervous system, and metabolic abnormalities; (4) not taken other vaginal preparation drugs 30 days before this trial; (5) voluntarily signed the informed consent. Subjects that were allergic to the postbiotics gel were excluded from this study (Figure 1).

2.2 Postbiotic gel preparation

The postbiotic gel used in this work was produced by Qingyitang Industrial Co., Ltd. (Yunnan, China). Probiotic strains were provided by the Lactic Acid Bacteria Collection Center of Inner Mongolia

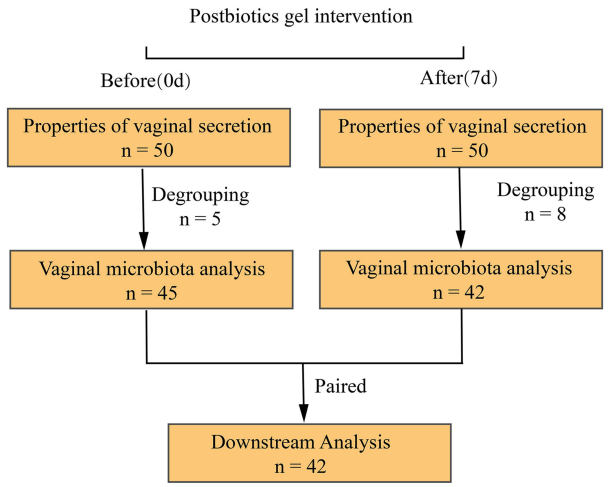

FIGURE 1
Trial design and enrollment information.

Agricultural University (Hohhot, China). The gel was prepared by mixing the raw materials (Table 1) with distilled water at 60°C, homogenize, sterilize, cool and ferment to pH 4.60 at 37°C. Citric acid was adjusted to pH 4.50 with deionized water. Kabo 940 was added and stirred until completely dissolved, followed by adding and mixing with triethanolamine, propylene glycol, PEG-90M, moisturizing gel, phenoxyethanol, and the fermentation solution in turn. The well-mixed gel was packaged as 3-gram tubes.

2.3 Postbiotic gel application, collection and analysis of vaginal secretions

Participants was registered to participate in this trial by providing basic demographic information (age: 35.8 ± 8.96) before the start of the trial. The gel was applied every night and avoided the menstrual period. To apply the gel, subjects first cleaned their hands and vulva with warm water, took a product, slightly lifted the hips in a supine position, slowly inserted the gel catheter containing the gel into the deep part of the vagina, pushed it into the pubic area with a pusher, and maintained the posture for about 15 minutes. The product was applied every night for seven days.

Sample collection is carried out by professional doctors. Fresh vaginal secretions were collected with sterile cottons before the start of medication and the next day of the last gel application. In order to obtain fresh samples, we collect the secretions of volunteers according to strict methods and submit them for examination as soon as possible. First of all, sterile cotton swabs were gently rotated in the subjects' posterior vaginal fornix to collect fresh vaginal secretions. After sampling, cottons with the samples were placed in test tubes containing physiological saline and stored at -80°C until further processing. At the same time, symptoms of subjects, including severity of vulvar itching and leucorrhea, color and odor of vaginal secretions were recorded. The biochemical and microbiological properties of vaginal secretion were determined in the laboratory within 30 minutes of sample collection by a medical professional. Hydrogen peroxide test was performed using a combined aerobic vaginitis/bacterial vaginosis five-item qualitative test kit (based on an enzyme-chemical reaction method; CSC Goldfield Diagnostics, Beijing, China). The presence of lactobacilli, fungi, and the cleanliness of collected samples were observed using a microscope (Olympus Corporation of Japan). The pH of the vaginal secretion was determined using pH test strips. All assessments were operated by professional doctors or personnel, and the data were entered and confirmed by more than two medical staff to ensure data consistency and accuracy. A total of 50 subjects completed the clinical assessment in the trial, and 47 subjects provided a complete sample (two vaginal secretions).

TABLE 1 The ratio of raw materials and the amount of probiotics inoculated.

Raw material	Content (%)
Skim milk powder (Fonterra, New Zealand)	5
Full-fat soybean powder (Dragon King of Heilongjiang Agricultural Reclamation)	3
Distilled water	92
Lacticaseibacillus paracasei ProSci-92	1×10^6CFU/mL
Lacticaseibacillus rhamnosus ProSci-109	1×10^6CFU/mL

2.4 Sequencing of bacterial microbiota in the vaginal samples

The metagenomic DNA in the collected vaginal secretions was extracted by QIAamp kit (Qiagen, Hilden, Germany), and the purity and concentration of the extracted DNA were detected by Nanodrop (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. Qualified DNA samples were amplified, targeting to the 16S rRNA V4 region using barcoded region-specific primers, 515F (GTGYCAGCMGCCGCGGTA) and 806R (GGACTACHVGGGTWTCTAAT). The amplification conditions were pre-denaturation at 95°C for 1 min; denaturation at 95°C for 30 s; annealing at 60°C for 40 s; extension at 72°C for 1 min, for a total of 30 cycles; terminal extension at 72°C for 7 min and termination at 4°C. Amplified samples were checked by agarose gel electrophoresis for the amplicon product size and purity. Follow-up analysis was only performed on amplicon products appearing as a single and bright band.

The samples that met the quality requirement were used for DNA library construction and sequenced using the Illumina novaseq PE250 platform. The original sequences were quality controlled and grouped by the sample nucleotide barcode. Microbial diversity analysis was performed using the QIIME platform (Caporaso et al., 2010) as follows: sequences aligned using PyNAST (Y. Zhang & Alekseyenko, 2017) to establish operational taxonomic units (OTUs) (Vu et al., 2018) according to the UCLUST two-step method. Selected representative sequences were taxonomically assigned by comparing against the SILVA database (Majaneva et al., 2015), the Greengene database (DeSantis et al., 2006), and the Ribosomal Database Project (Release 11.5) database (Maidak et al., 1996).

2.5 Statistical analyses

For α diversity analysis, QIIME (version 1.9.1) was used to calculate the Shannon index, chao1 index, and Simpson index. The Shannon curve and the observed species number curve were plotted using R (version 4.2.1), which were used to assess the bacterial diversity of each sequenced sample and the sequencing depth. Wilcoxon tests were used to evaluate the α diversity between samples collected at the two time points. β diversity (weighted and unweighted Unifrac) was calculated by R packages (vegan), and principal coordinate analysis (PCoA) was used for descending presentation. Association between β diversity and study groups was assessed using a non-parametric analysis of similarities (Adonis, vegan R package) with 999 permutations. Followed by comparative analysis to identify significant differential marker bacteria of treatment (cut-off: $P < 0.05$; Wilcoxon test). PICRUST2 (Douglas et al., 2020) was used for functional annotation and STAMP (Parks et al., 2014) was used for data visualization.

3 Results

3.1 Improvement in subjects' clinical indicators after postbiotic gel application

In our study, 50 people completed clinical indicators, including 45 people who completed vaginal discharge collection before the intervention and 42 people after the intervention. After matching, the per-protocol population was 42, who completed the process of clinical information collection and provision of vaginal secretion for microbiota sequencing analysis before and after using the postbiotic gel. The vaginal sample of a healthy woman is transparent or milky white, with no odor and in a small amount. A large amount of vaginal secretion that is thin, homogeneous, and of fishy odor is indicative of vaginal inflammation (Amsel et al., 1983). This study compared these properties of subjects' vaginal secretions before and after use of the postbiotic gel, and found that properties of subjects' vaginal secretions (e.g., color, clarity, odor) improved significantly after using the postbiotic gel (Table 2).

Vulvar pruritus is one of the reference indicators for evaluating vaginitis. Three among the 50 patients in this study complaint about vaginal itching at the start of the trial, and the symptom remained only in one subject after using the postbiotic gel. A high level of vaginal secretions cleanliness (the cleanliness rating is closer to IV) and positive reaction in hydrogen peroxide test indicate poor BV or at least poor vaginal health. Initially, 47 patients showed a positive result for hydrogen peroxide, and 18 (remission rate = 38.3%) of them showed a negative hydrogen peroxide result after using the gel. Moreover, 29 people had a cleanliness grade of III or IV, and most (18) of them showed significant improvement after using the postbiotic gel. These data suggested that applying the postbiotic gel for a week could improve BV and vaginal health.

3.2 Changes in the α-diversity of vaginal microbiota after the intervention

A total of 5,479,608 high-quality sequences were obtained from 87 (Before: n = 45; After: n = 42) sequencing samples. The rarefaction curves of the Shannon diversity (Figure 2A), but not the number of observed species (Figure 2B) leveled off, suggesting that most of the bacterial diversity was already captured although new species could still be found. Therefore, this sequencing depth was adequate to reflect a representative diversity of the vaginal microbiota.

In order to study the difference between the two groups of samples, the samples were screened, and the samples that could not be paired were excluded, and a total of 42 pairs of samples were obtained. The chao1, number of observed species, Shannon, and Simpson indexes of the vaginal microbiota of subjects were analyzed, and decreasing trends were observed in all four measured diversity indexes after using the postbiotic gel, though the differences were not statistically significant (Figure 2C). The decrease in species diversity and abundance could be attributed to the effect of application of the postbiotic gel in shaping the vaginal environment for bacterial growth.

3.3 β-diversity analysis, identification of differential bacteria before/after the gel intervention

Changes in the β-diversity of the vaginal microbiota was analyzed by PCoA (weighted and un-weighted Unifrac), which revealed no significant differences before/after the gel intervention ($P > 0.05$; Figures 3A, B). The taxonomic analysis of the vaginal microbiota composition revealed five dominant phyla. After applying the gel, the vaginal microbiota showed increased levels in Actinobacteria and Fusobacteria, and decreased levels in Proteobacteria and Bacteroidetes, though the differences were not statistically significant ($P > 0.05$; Figure 3C). At the genus level (Figure 3D), the major genera (those > 1% average relative abundance before and after

TABLE 2 Changes in vaginal secretion properties and biochemical parameters after the use of postbiotic gel.

Index	Amount of leucorrhea		Leucorrhea traits		Leucorrhea smell		H_2O_2		Cleanliness	
	unchanged	19 cases	dilute → thick	10 cases	none → none	45 cases	positive → positive	29 cases	II → II	19 cases
	reduce	29 cases	dilute → thick	1 case	yes → none	5 cases	positive → negative	18 cases	II → III	2 cases
	increase	2 cases	thick → dilute	23 cases			negative → negative	2 cases	III → II	16 cases
			thick → thick	16 cases			negative → positive	1 case	III → III	11 cases
									IV → II	2 cases
Remission rate	64.44%		58.97%		100%		38.30%		62.07%	

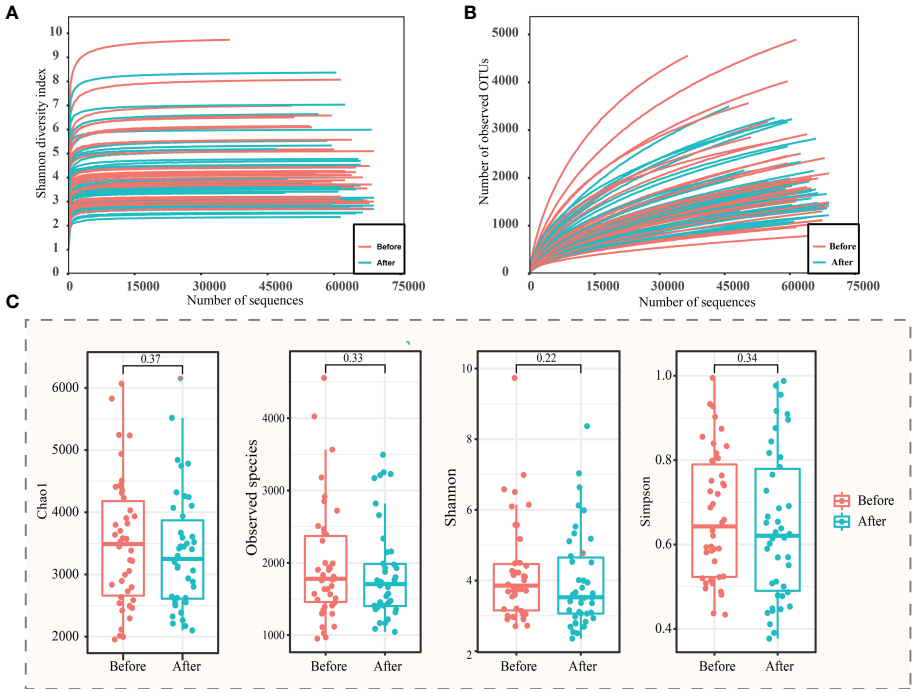

FIGURE 2
α-diversity analysis of vaginal microbiota before and after the use of the postbiotic gel. Rarefaction curves of **(A)** Shannon diversity and **(B)** observed operational taxonomic units (OTUs). **(C)** Box plots of alpha diversity (chao1, observed species, Shannon and Simpson diversity indexes) before and after using postbiotic gel.

using the postbiotic gel) included *Lactobacillus* (71.56%), *Gardnerella* (6.02%), *Prevotella* (3.30%), *Streptococcus* (1.62%), and *Atopobium* (2.89%). After using the postbiotic gels, the relative abundances of some pathogenic bacteria, including *Gardnerella* (Figure 3E), *Streptococcus*, and *Prevotella*, decreased, while the relative abundance of *Atopobium* increased. The relative abundance of *Streptococcus* decreased significantly after the gel use ($P = 0.0001$) (Figure 3F), while no significant changes were observed in the genus *Atopobium* ($P = 0.0596$; Table S1).

We used microscopy to analyze *Lactobacillus* in the secretions and found that *Lactobacillus* was not observed before the gel intervention and 10 samples in which *Lactobacillus* was found after the intervention, but only 5 of these 10 cases were sequenced by Illumina both before and after the use of the gel. Through the analysis of the relative content of *Lactobacillus* in 5 cases, it was found that the relative content of *Lactobacillus* in 4 samples increased in varying degrees. It is worth mentioning that among all sequenced samples (Before: n = 42; After: n = 42), the content of *Lactobacillus* in vaginal secretions of 24 patients increased (Figure 3G), accounting for 57% of the total number of patients, it can be seen that exogenous gel can increase the number of lactobacilli vaginal.

3.4 Changes in the predicted function of the vaginal microbiota after postbiotic gel application

We then used PICRUSt2 to predict functional changes in the vaginal microbiota after using the postbiotic gel (Figure 4), and significant changes were found in the encoded function of the vaginal microbiota, including pathways of metabolism, genetic information processing, and organic system (Table S2). On the secondary level of KEGG pathway, the vaginal microbiota was significantly enriched in the pathways of biosynthesis of other secondary metabolites, metabolism of terpenoids and polyketides, and endocrine system and digestive system, which were significantly decreased after using the gel ($P < 0.05$; Table S2). On the tertiary level of KEGG pathway, eight pathways exhibited significant decreases after using the postbiotic gel ($P < 0.05$; Figure 4; Table S2). These data supported that the application of the postbiotic gel could modulate the predicted functional pathways of the vaginal microbiota, which might be associated with the symptom improvement.

3.5 Correlation between dominant microbiota and clinical features of the vaginal secretion

According to our clinical observation, we selected the common clinical symptoms and signs, and referred to the symptom scoring standard in the Guidelines for Clinical Research on New Chinese Medicines (for Trial Implementation). For evaluating the amount of secretion: a small amount was scored as 2, a medium amount was scored as 1, and a large amount was scored as 0. For evaluating the nature of secretion: a thin amount was scored as 1, and a thick amount was scored as 0. For evaluating the odor of secretion: an odorless amount was scored as 1, and an odorous amount was scored as 0. For evaluating the color of secretion: a clear color was scored as

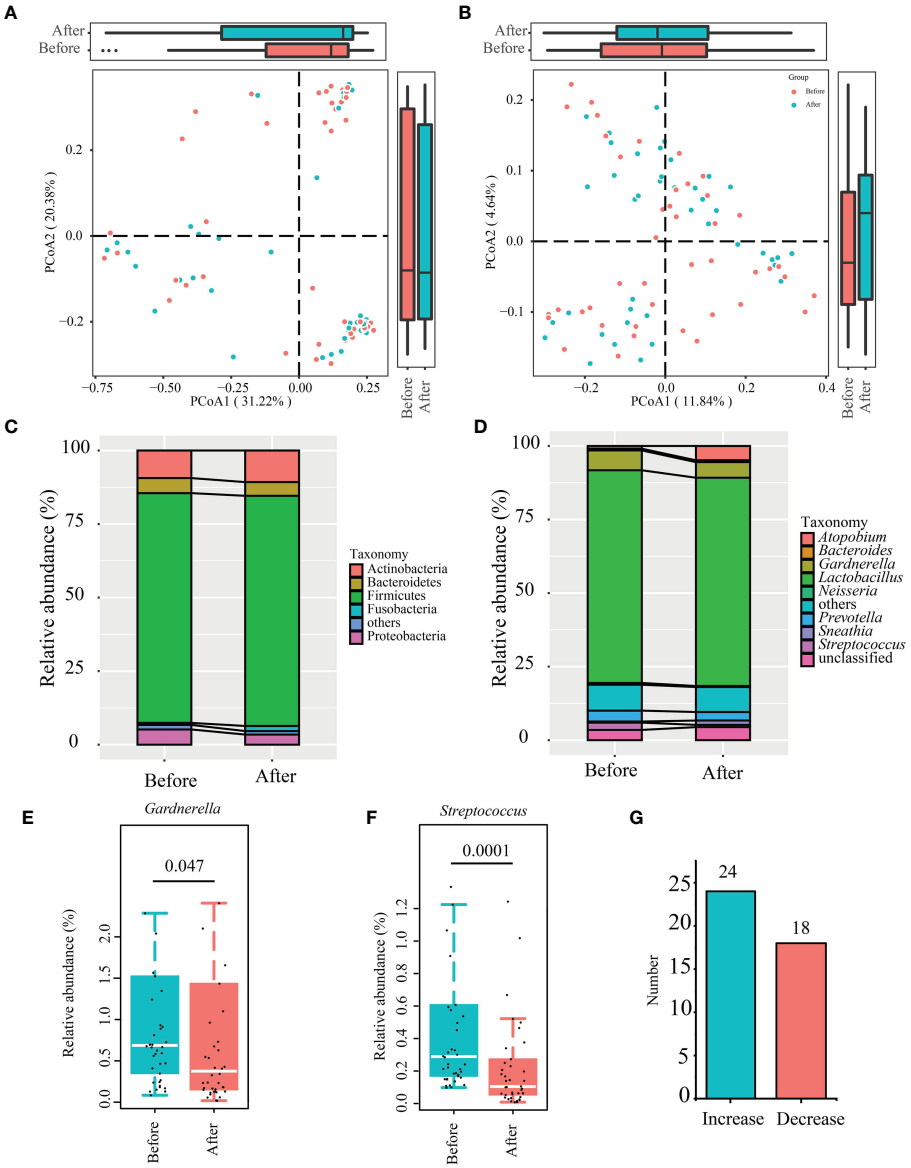

FIGURE 3
Changes in the β-diversity of vaginal microbiota and differential abundant bacteria identified after using the postbiotic gel. **(A, B)** Principal co-ordinates analysis (PCoA; weighted and unweighted unifrac) of subjects' vaginal microbiota. **(C)** Phylum- and **(D)** genus-level taxonomic profiles of subjects' bacterial vaginal microbiota before and after applying the postbiotic gel. **(E, F)** Box plots of the differential abundant bacterial genera, *Gardnerella* and *Streptococcus* before and after using the postbiotic gel. **(G)** The number of people with changes in *Lactobacillus* content.

3, a light yellow color was scored as 2, a yellow color was scored as 1, and a green color was scored as 0. Then we used Spearman's correlation analysis to analyze the association between the dominant bacterial genera and clinical features of the collected vaginal samples (Figure 5; Table S3). Interestingly, we found significant negative correlations between *Lactobacillus* and a number of (potential) pathogens, i.e., *Gardnerella*, *Streptococcus*, *Prevotella*, *Atopobium* ($P < 0.001$, $r = -0.4937$; $P < 0.001$, $r = -0.4165$; $P < 0.001$, $r = -0.6267$; $P = 0.0043$, $r = -0.3082$, respectively). *Streptococcus* showed a weak positive correlation with *Prevotella* ($P = 0.045$, $r = 0.22$), but negative correlations with the amount and characteristics of vaginal secretion ($P = 0.0061$, $r = -0.2968$; $P = 0.0178$, $r = -0.2579$, respectively; Figure 5).

4 Discussion

The colonization of lactic acid bacteria plays an important role in maintaining vaginal homeostasis by inhibiting other pathogens, and the colonization of anaerobic bacteria is often related to the occurrence of BV (Fredricks et al., 2005). The main reason of the onset of BV is vaginal dysbiosis characterized by a decrease in lactobacilli, leading to the proliferation of pathogenic bacteria such as *Gardnerella vaginalis*, *Prevotella* spp., and *Atopobium vaginae* (Latham-Cork et al., 2021). Antibiotics are traditionally used in treating BV, but antibiotic application can easily disrupt the homeostasis of the vaginal microecology and the endogenous vaginal microflora. Thus, reinfection is common after treating BV with antibiotics. To find a

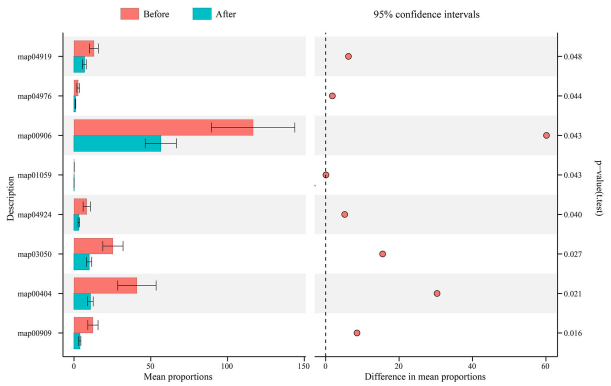

FIGURE 4
Changes in the functional microbiota predicted by PICRUSt after applying the postbiotic gel. Eight significant differential KEGG pathways (t-test; $P < 0.05$) were identified before (shown in red) and after (shown in blue) applying the postbiotic gel.

safer and more effective treatment, probiotics have been applied. In this study, we investigated the symptom alleviation effect of applying a postbiotic gel in treating BV. We assessed the changes in the clinical features of vaginal secretion and microbiota in patients with BV after applying postbiotics.

A randomized, double-blind, placebo-controlled trial evaluated the routine treatment of BV or vulvovaginal candidiasis for five days with probiotic capsule, revealing improvement in symptoms, which was accompanied by lactobacilli colonization of the vagina, reduced recurrence rate, and reduced odor in the vaginal discharge, which is consistent with our findings (Ehrström et al., 2010). Furthermore,

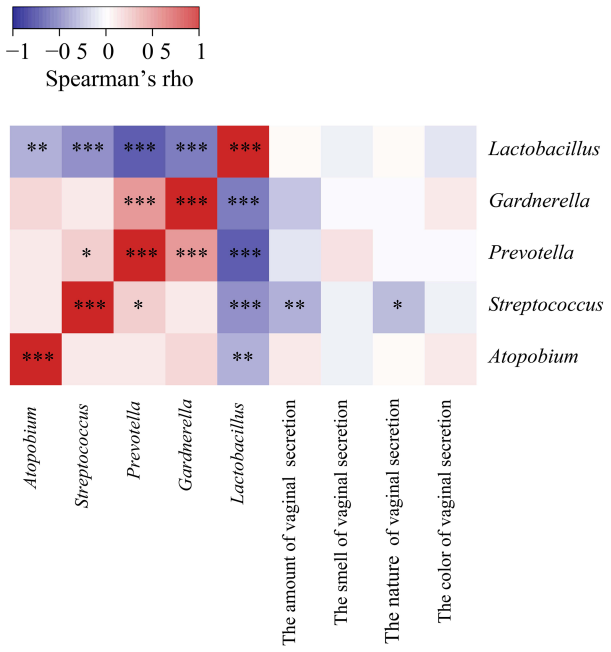

FIGURE 5
Spearman's Correlation heat maps showing association between dominant bacteria and properties of subjects' vaginal secretion. The color scale shows the strength of correlation (ranging from 1 to -1, representing strong positive to strong negative correlation). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Cohen et al. (2020) found that applying Lactin-V (a metronidazole vaginal containing *Lactobacillus crispatus* CTV-05) for 11 weeks was effective in reducing the recurrence rate of BV in 228 patients (Cohen et al., 2020). Thus, postbiotic gel is useful in maintaining vaginal health, managing BV, and reduce the recurrent rate.

In this study, we found no significant difference in the α diversity of vaginal microbiota showed a non-significant reduction after using the postbiotic gel. It is important to note that, contrasting to the gut microbiota, a healthy vaginal microbiota has a low bacterial diversity. A high vaginal microbiota diversity increases the probability of BV (Zevin et al., 2016). In addition, the dominance of vaginal lactobacilli is one of the criteria for vaginal health. Consistent to the dominance in lactobacilli observed in this study, Witkin et al. (2013) also showed that the vaginal microbiota comprises a high proportion (71.3%) of *Lactobacillus* (Witkin et al., 2013). Our results showed that applying the postbiotic gel increased the relative abundance of lactobacilli in the vaginal secretion of 24 patients (corresponding to 57% of the total number of patients). Lactobacilli can inhibit the growth of pathogenic microorganisms by producing a variety of secondary metabolites with antibacterial activity, such as lactic acid, hydrogen peroxide, and biosurfactants (Borges et al., 2014). Thus, they play an important role in maintaining the stability of vaginal microenvironment and microbiota.

Gardnerella is a conditional pathogen in vaginitis. The overgrowth of *Gardnerella* causes a vaginal dysbiosis, leading to the occurrence of vaginal diseases, such as BV. In the present study, the mean relative abundance of vaginal *Gardnerella* spp. was significantly reduced (from 6.7% to 5.3%; $P < 0.05$) after using the postbiotic gel. *Gardnerella* can adhere to vaginal epithelial cells, forming a dense biofilm, which can enhance the bacterial tolerance to high concentrations of antibacterial molecules like hydrogen peroxide and lactic acid (Hardy et al., 2017). Moreover, the biofilm formation also enhances the resistance of vaginal pathogens to the host mucosal immune defense, favoring problems such as recurrent BV (He et al., 2021). Notably, *Gardnerella* is also the main indicator and pathogen of BV. Therefore, reducing the colonization of *Gardnerella* may be one way to improve BV. The reduction in *Gardnerella* supported that the current probiotic is effective in pathogen inhibition.

Then, a correlation analysis was performed between the dominant genera of the vaginal microbiota and properties of subjects' vaginal secretions. Again, we found a significant negative association between lactobacilli and potential pathogens like *Gardnerella*, *Atopobium*, and *Prevotella*. It is worth mentioning that BV is often associated with the presence of high loads of *Atopobium vaginae* and/or *Gardnerella vaginalis* (Menard et al., 2008), and that there is a synergistic effect between several bacterial vaginitis pathogens including *Gardnerella*, *Atopobium*, and *Prevotella*. The initial biofilm formed by *Gardnerella* vaginalis in the vaginal epithelium facilitates the attachment and colonization of other pathogenic bacteria, such as *Atopobium*, thus producing more complex biofilms of multiple bacteria, leading to the occurrence of refractory vaginitis (Arroyo-Moreno et al., 2022). In addition, our study found that the predicted function of the vaginal microbiota was improved after using the postbiotics.

In conclusion, our study showed that applying the current postbiotic gel could improve the patients' symptoms of BV, and the symptom improvement was accompanied by significant changes in

patients' vaginal microbiota, characterized by an increase in lactobacilli and a reduction in multiple potential vaginal pathogens. Our data supported that postbiotics application could improve vaginal health and ease BV. Our findings have a high application value in clinical practice.

5 Limitations

There are some limitations in our study. Firstly, the number of subjects included in this trial. was small, and the number of subjects should be increased in subsequent studies with the inclusion of a placebo control group of healthy subjects for a baseline of vaginal microbiota in comparison with subjects with BV. Secondly, 16S rRNA gene amplicon sequencing only represents the genus-level genome without providing any information on gene or protein expression, which would provide more functional information (M. T. France et al., 2022b). In future studies, these aspects should be considered to improve the trial design. Moreover, the trial design should also be expanded to analyze changes in the functional microbiota to provide insights into the mechanism of symptom relief.

Data availability statement

All sequence data generated in this study were submitted to the MG-RAST database under the ID number mgp95926.

Ethics statement

The studies involving human participants were reviewed and approved by independent committee members. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XS: Formal analysis, data curation, visualization, writing of the original draft. LX: Clinical trial implementation, specimen collection. ZQZ: Conceptualization, design of methodology. YTY and PXL: Formal analysis, software testing and verification. TM and SG: Supervision of clinical trial, manuscript revision. L-YK: Writing, critical evaluation and revision of the original draft, resource provision. ZHS: Conceived and designed the experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors ZQZ, YTY, and PXL were employed by company Qingyitang Industrial Co.,.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1114364/full#supplementary-material

SUPPLEMENTARY TABLE 1
Differential bacterial genera identified before and after using the postbiotic gel.

SUPPLEMENTARY TABLE 2
Differential pathway identified before and after using the postbiotic gel.

SUPPLEMENTARY TABLE 3
Spearman correlation analysis between vaginal discharge and differential genera.

References

Amsel, R., Totten, P. A., Spiegel, C. A., Chen, K. C., Eschenbach, D., and Holmes, K. K. (1983). Nonspecific vaginitis. diagnostic criteria and microbial and epidemiologic associations. *Am. J. Med.* 74 (1), 14–22. doi: 10.1016/0002-9343(83)91112-9

Armstrong, E., Hemmerling, A., Miller, S., Burke, K. E., Newmann, S. J., Morris, S. R., et al. (2022). Metronidazole treatment rapidly reduces genital inflammation through effects on bacterial vaginosis-associated bacteria rather than lactobacilli. *J. Clin. Invest.* 132 (6), e152930. doi: 10.1172/jci152930

Arroyo-Moreno, S., Cummings, M., Corcoran, D. B., Coffey, A., and McCarthy, R. R. (2022). Identification and characterization of novel endolysins targeting gardnerella vaginalis biofilms to treat bacterial vaginosis. *NPJ Biofil. Microbio.* 8 (1), 29. doi: 10.1038/s41522-022-00285-0

Borges, S., Silva, J., and Teixeira, P. (2014). The role of lactobacilli and probiotics in maintaining vaginal health. *Arch. Gynecol. Obstet.* 289 (3), 479–489. doi: 10.1007/s00404-013-3064-9

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7 (5), 335–336. doi: 10.1038/nmeth.f.303

Chen, T., Xia, C., Hu, H., Wang, H., Tan, B., Tian, P., et al. (2021). Dysbiosis of the rat vagina is efficiently rescued by vaginal microbiota transplantation or probiotic combination. *Int. J. Antimicrob. Agents* 57 (3), 106277. doi: 10.1016/j.ijantimicag.2021.106277

Chen, L., Zhang, Q., Wang, Y., Zhang, K., and Zou, J. (2019). Comparing dental plaque microbiome diversity of extrinsic black stain in the primary dentition using illumina MiSeq sequencing technique. *BMC Oral. Health* 19 (1), 269. doi: 10.1186/s12903-019-0960-9

Cohen, C. R., Wierzbicki, M. R., French, A. L., Morris, S., Newmann, S., Reno, H., et al. (2020). Randomized trial of lactin-V to prevent recurrence of bacterial vaginosis. *N. Engl. J. Med.* 382 (20), 1906–1915. doi: 10.1056/NEJMoa1915254

Delgado-Diaz, D. J., Jesaveluk, B., Hayward, J. A., Tyssen, D., Alisoltani, A., Potgieter, M., et al. (2022). Lactic acid from vaginal microbiota enhances cervicovaginal epithelial barrier integrity by promoting tight junction protein expression. *Microbiome* 10 (1), 141. doi: 10.1186/s40168-022-01337-5

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72 (7), 5069–5072. doi: 10.1128/aem.03006-05

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38 (6), 685–688. doi: 10.1038/s41587-020-0548-6

Ehrström, S., Daroczy, K., Rylander, E., Samuelsson, C., Johannesson, U., Anzén, B., et al. (2010). Lactic acid bacteria colonization and clinical outcome after probiotic supplementation in conventionally treated bacterial vaginosis and vulvovaginal candidiasis. *Microbes Infect.* 12 (10), 691–699. doi: 10.1016/j.micinf.2010.04.010

France, M., Alizadeh, M., Brown, S., Ma, B., and Ravel, J. (2022b). Towards a deeper understanding of the vaginal microbiota. *Nat. Microbiol.* 7 (3), 367–378. doi: 10.1038/s41564-022-01083-2

France, M. T., Fu, L., Rutt, L., Yang, H., Humphrys, M. S., Narina, S., et al. (2022a). Insight into the ecology of vaginal bacteria through integrative analyses of metagenomic and metatranscriptomic data. *Genome Biol.* 23 (1), 66. doi: 10.1186/s13059-022-02635-9

Fredricks, D. N., Fiedler, T. L., and Marrazzo, J. M. (2005). Molecular identification of bacteria associated with bacterial vaginosis. *N. Engl. J. Med.* 353 (18), 1899–1911. doi: 10.1056/NEJMoa043802

Happel, A. U., Kullin, B., Gamieldien, H., Wentzel, N., Zauchenberger, C. Z., Jaspan, H. B., et al. (2020). Exploring potential of vaginal lactobacillus isolates from south African women for enhancing treatment for bacterial vaginosis. *PloS Pathog.* 16 (6), e1008559. doi: 10.1371/journal.ppat.1008559

Hardy, L., Cerca, N., Jespers, V., Vaneechoutte, M., and Crucitti, T. (2017). Bacterial biofilms in the vagina. *Res. Microbiol.* 168 (9-10), 865–874. doi: 10.1016/j.resmic.2017.02.001

He, Y., Na, R., Niu, X., Xiao, B., and Yang, H. (2021). Lactobacillus rhamnosus and lactobacillus casei affect various stages of gardnerella species biofilm formation. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.568178

Latham-Cork, H. C., Walker, K. F., Thornton, J. G., Gunnarsson, O. S., Säfholm, A., Cardell, M., et al. (2021). A novel non-antimicrobial treatment of bacterial vaginosis: An open label two-private centre study. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 256, 419–424. doi: 10.1016/j.ejogrb.2020.11.059

Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J., and Woese, C. R. (1996). The ribosomal database project (RDP). *Nucleic Acids Res.* 24 (1), 82–85. doi: 10.1093/nar/24.1.82

Majaneva, M., Hyytiäinen, K., Varvio, S. L., Nagai, S., and Blomster, J. (2015). Bioinformatic amplicon read processing strategies strongly affect eukaryotic diversity and the taxonomic composition of communities. *PloS One* 10 (6), e0130035. doi: 10.1371/journal.pone.0130035

Menard, J. P., Fenollar, F., Henry, M., Bretelle, F., and Raoult, D. (2008). Molecular quantification of gardnerella vaginalis and atopobium vaginae loads to predict bacterial vaginosis. *Clin. Infect. Dis.* 47 (1), 33–43. doi: 10.1086/588661

Nugent, R. P., Krohn, M. A., and Hillier, S. L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J. Clin. Microbiol.* 29 (2), 297–301. doi: 10.1128/jcm.29.2.297-301.1991

Oakley, B. B., Fiedler, T. L., Marrazzo, J. M., and Fredricks, D. N. (2008). Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Appl. Environ. Microbiol.* 74 (15), 4898–4909. doi: 10.1128/aem.02884-07

Onderdonk, A. B., Delaney, M. L., and Fichorova, R. N. (2016). The human microbiome during bacterial vaginosis. *Clin. Microbiol. Rev.* 29 (2), 223–238. doi: 10.1128/cmr.00075-15

Parks, D. H., Tyson, G. W., Hugenholtz, P., and Beiko, R. G. (2014). STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30 (21), 3123–3124. doi: 10.1093/bioinformatics/btu494

Rostok, M., Hütt, P., Rööp, T., Smidt, I., Štšepetova, J., Salumets, A., et al. (2019). Potential vaginal probiotics: Safety tolerability and preliminary effectiveness. *Benef. Microbes* 10 (4), 385–393. doi: 10.3920/bm2016.0123

Russo, R., Edu, A., and De Seta, F. (2018). Study on the effects of an oral lactobacilli and lactoferrin complex in women with intermediate vaginal microbiota. *Arch. Gynecol. Obstet.* 298 (1), 139–145. doi: 10.1007/s00404-018-4771-z

Salminen, S., Collado, M. C., Endo, A., Hill, C., Lebeer, S., Quigley, E. M. M., et al. (2021). The international scientific association of probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. *Nat. Rev. Gastroenterol. Hepatol.* 18 (9), 649–667. doi: 10.1038/s41575-021-00440-6

Schellekens, H., Torres-Fuentes, C., van de Wouw, M., Long-Smith, C. M., Mitchell, A., Strain, C., et al. (2021). Bifidobacterium longum counters the effects of obesity: Partial successful translation from rodent to human. *EBioMedicine* 63, 103176. doi: 10.1016/j.ebiom.2020.103176

Sun, H., Zhao, F., Liu, Y., Ma, T., Jin, H., Quan, K., et al. (2022). Probiotics synergized with conventional regimen in managing parkinson's disease. *NPJ Parkinsons. Dis.* 8 (1), 62. doi: 10.1038/s41531-022-00327-6

Vicariotto, F., Mogna, L., and Del Piano, M. (2014). Effectiveness of the two microorganisms lactobacillus fermentum LF15 and lactobacillus plantarum LP01 formulated in slow-release vaginal tablets in women affected by bacterial vaginosis: A pilot study. *J. Clin. Gastroenterol.* 48 Suppl 1, S106–S112. doi: 10.1097/mcg.0000000000000226

Vu, D., Georgievska, S., Szoke, S., Kuzniar, A., and Robert, V. (2018). fMLC: Fast multi-level clustering and visualization of large molecular datasets. *Bioinformatics* 34 (9), 1577–1579. doi: 10.1093/bioinformatics/btx810

Wallace, C., Sinopoulou, V., Gordon, M., Akobeng, A. K., Llanos-Chea, A., Hungria, G., et al. (2022). Probiotics for treatment of chronic constipation in children. *Cochrane Database Syst. Rev.* 3 (3), Cd014257. doi: 10.1002/14651858.CD014257.pub2

Walter, K. (2022). Premenopausal vaginitis. *JAMA* 327 (22), 2255–2255. doi: 10.1001/jama.2022.9256

Wang, Y., She, M., Liu, K., Zhang, Z., and Shuang, Q. (2020). Evaluation of the bacterial diversity of inner Mongolian acidic gruel using illumina MiSeq and PCR-DGGE. *Curr. Microbiol.* 77 (3), 434–442. doi: 10.1007/s00284-019-01848-9

Witkin, S. S., Mendes-Soares, H., Linhares, I. M., Jayaram, A., Ledger, W. J., and Forney, L. J. (2013). Influence of vaginal bacteria and d- and l-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: Implications for protection against upper genital tract infections. *mBio* 4 (4), e00460–13. doi: 10.1128/mBio.00460-13

Xiao, B., A, D., Qin, H., Mi, L., and Zhang, D. (2022). Correlation analysis of vaginal microbiome changes and bacterial vaginosis plus vulvovaginal candidiasis mixed vaginitis prognosis. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.860589

Zevin, A. S., Xie, I. Y., Birse, K., Arnold, K., Romas, L., Westmacott, G., et al. (2016). Microbiome composition and function drives wound-healing impairment in the female genital tract. *PloS Pathog.* 12 (9), e1005889. doi: 10.1371/journal.ppat.1005889

Zhang, Y., and Alekseyenko, A. V. (2017). Phylogenic inference using alignment-free methods for applications in microbial community surveys using 16s rRNA gene. *PloS One* 12 (11), e0187940. doi: 10.1371/journal.pone.0187940

Zhang, Z., Zhang, D., Xiao, B. B., Zhang, R., Bai, H. H., Dong, H. Y., et al. (2018). [Primary study on the relationship between high-risk HPV infection and vaginal cervical microbiota]. *Zhonghua. Fu. Chan. Ke. Za. Zhi.* 53 (7), 471–480. doi: 10.3760/cma.j.issn.0529-567x.2018.07.006

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EDITED BY
Zhangran Chen,
Shenzhen Wedge Microbiology Research Co. LTD, China

REVIEWED BY
Maria D'Accolti,
University of Ferrara, Italy
Liqing Wei,
Wuhan University, China
Yuzhu Dai,
The 903rd Hosipital of the PLA, China

*CORRESPONDENCE
Yuan Li
liyuan1596215@163.com
Jian Wu
wujianglinxing@163.com

†These authors have contributed equally to this work

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Vulvovaginal candidiasis and vaginal microflora interaction: Microflora changes and probiotic therapy

Zhongwen Sun[1†], Xinnuo Ge[2†], Bo Qiu[3†], Ze Xiang[2], Chun Jiang[4], Jian Wu[4*] and Yuan Li[5*]

[1]Department of Medical Technology, Suzhou Vocational Health College, Suzhou, Jiangsu, China, [2]Zhejiang University School of Medicine, Hangzhou, Zhejiang, China, [3]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China, [4]Department of Clinical Laboratory, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Gusu School, Nanjing Medical University, Suzhou, Jiangsu, China, [5]Departments of Cardiology, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Gusu School, Nanjing Medical University, Suzhou, Jiangsu, China

Vaginal microbiome is mutually beneficial to the host and has a significant impact on health and disease. *Candida* species, including *Candida albicans*, are part of the mucosal flora of most healthy women. Under suitable conditions, they can live in the vulvovaginal mucosa, resulting in symptomatic vulvovaginal candidiasis (VVC). Based on the analysis of 16S ribosomal RNA gene sequences, great progress has been made in exploring the composition and structure of vaginal bacterial community. Moreover, researchers have conducted several studies on whether vaginal microbiome will change during VVC infection. In addition, it has been reported that vaginal colonization of probiotics in vaginal microorganisms, especially *Lactobacillus*, can effectively reduce the risk of VVC and treat VVC. This review aims to summarize the changes of vaginal microflora during VVC infection, and further point out the possibility of using lactic acid bacteria as probiotics to treat VVC, so as to reduce the adverse consequences of VVC infection and reduce the expensive treatment cost.

KEYWORDS

vulvovaginal candidiasis, vaginal infection, microbial changes, *Lactobacillus*, treatment

Introduction

Bacteria are the largest group of all organisms, which first appeared about 3.8 billion years ago, and nucleolus is one of their most obvious signs (Mojzsis et al., 1996). Fungi have true nuclei and complete organelles, so they are also called eukaryotic cell type microorganisms (Lopez-Garcia and Moreira, 2020). The human microbiome is made up of archaea, bacteria, viruses, and fungi that form a highly complex web of interactions with each other and their hosts (Wang et al., 2015; Wu et al., 2020). The host and the microbial community have co-

evolved an immune system to prevent the colonization of the foreign microorganisms in the body (Wu et al., 2019). Researchers have used a variety of methods, including metabolomics, proteomics, transcriptomics and metagenomics, to verify that microbial communities are dynamic, interactive and complex organic whole (Chen et al., 2021; Xiang et al., 2022).

The impact of microbial flora in the human body has aroused extensive concern. It not only plays an important role in the intestine, which is widely studied nowadays, but also gradually functions in other organ systems (Champer et al., 2018; Khanna et al., 2022). Nowadays, researchers pay more and more attention to women's health, especially the vaginal microbiota, since the vagina has a huge microecosystem containing billions of species of microorganisms (Witkin and Forney, 2020). In recent years, tremendous advances have been made in exploring the composition and structure of vaginal bacterial community using the method based on analysis of 16S ribosomal RNA (rRNA) gene sequences (Chen et al., 2017). In the case of bacterial or fungal infections, changes in the vaginal microbiome have also been reported (Onderdonk et al., 2016; Chen et al., 2021).

In addition to the widely studied bacterial vaginosis (BV), vulvovaginal candidiasis (VVC) has been a hot topic, which is a multifactor infectious disease of women's lower reproductive tract, mainly caused by *Candida albicans*, resulting in pathological inflammation (Farr et al., 2021). In a study that collected 649 separate strains of VVC patients in China, researchers found that *Candida albicans* was the dominant pathogen of VVC, but the proportion of non-*Candida albicans* infection was also increasing (Pang et al., 2022).

This review aims to describe changes in the vaginal microbiome during VVC, propose the possible associations between VVC and vaginal microbiome changes, and summarize the possibility of using this association to treat and defend VVC to reduce adverse health outcomes and treatment costs.

Vaginal microbiota in healthy women

The native microbiota in the vaginal environment is thought to be symbiotic with the host (Ma et al., 2012). The technology for assessing human microbial diversity has gradually progressed. Nowadays, scientists have successfully identified different bacterial communities in the vagina of women of four races using advanced high-throughput methods and analyzed species composition through 16S rRNA gene sequencing (Di Bella et al., 2013). The vaginal bacterial communities of these women can be roughly divided into five types: the first four are mainly composed of *Lactobacillus crispatus*, *Lactobacillus gasseri, Lactobacillus iners, and Lactobacillus jensenii*, while the fifth bacterial community is relatively specific, showing a low proportion of *Lactobacillus* and a high proportion of strictly anaerobic organisms (Ravel et al., 2011). Therefore, *Lactobacillus* are considered the most common microorganisms isolated from the vagina of healthy people, including *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jensenii* (Chee et al., 2020). In several studies, these vaginal *Lactobacillus* can be used to prevent the invasion of pathogens (Petrova et al., 2017; Kalia et al., 2020).

In addition, fungi, especially *Candida*, are believed to exist in the vaginal mucosa as symbiotes, forming a complex vaginal ecosystem with other bacteria (Gow and Hube, 2012; Hall and Noverr, 2017). The defense function of fungi plays a role through various mechanisms, including lowering vaginal pH, producing bioactive compounds, competing for nutrients and adhesion sites, and regulating host immune responses (Parolin et al., 2015; Calonghi et al., 2017; Parolin et al., 2018; Younes et al., 2018).

Of course, it cannot be ignored that the vaginal microbiome is variable. Vaginal microbiome varies among individuals, and these differences are caused by differences in sexual habits, menstrual hygiene habits (Noyes et al., 2018), flushing habits (Schwebke et al., 1999), chronic stress (Culhane et al., 2002), geography (Gupta et al., 2017), socioeconomic status, psychosocial pressure, community characteristics (Paul et al., 2008), and other factors. Moreover, vaginal pH also varies due to the different compositions in certain populations. Recent studies have found that a small percentage of asymptomatic healthy women have low levels of *Lactobacillus* in their vaginas, but they include a variety of facultative or strictly anaerobic bacteria with a slightly higher pH (5.3-5.5), which is similar to the fifth bacterial community described above. When these women did not have the disease, their vaginal bacterial community was considered normal, while the abnormal type of vaginal microbiome may be strongly associated with symptomatic bacterial vaginosis (Zhou et al., 2007; Zhou et al., 2010; Denning et al., 2018). Furthermore, vaginal microorganisms are a dynamic ecosystem of more than 200 bacteria, and the same individuals may also be significantly different from their previous performance (Chen et al., 2017). There is some evidence that the vaginal microbiome changes throughout a woman's life and therefore has an important impact on the quality of life from newborn to post-menopausal age.

The changes of the vaginal microbiome include the decrease in the abundance of *Lactobacillus* and the increase in the abundance of facultative and anaerobic organisms, which can make the host vulnerable to a variety of diseases, such as low birth weight and increased bacterial infection risk (Jayaram et al., 2020). Therefore, the physiological state of the vaginal environment is of great significance for the health and reproduction of the host (Amabebe and Anumba, 2018).

To sum up, based on the variable and important characteristics of vaginal microbiome, we strongly advocate a more precise definition of the bacterial community in healthy women, which should fully consider the differences among individuals. The precise definition of a healthy vaginal microbiome can be used to diagnose disease faster and more accurately when the vaginal microbiome changes.

Vaginal microbial changes during VVC

The disruption of vaginal ecosystem balance can cause pathogen overgrowth, which will lead to more complex vaginal infections, such as BV, sexually transmitted infections (STIs), and VVC (Chee et al., 2020). Among them, VVC is defined as a symptom of inflammation and excessive overgrowth of *Candida*, especially *Candida albicans* (Sobel, 2007). *Candida albicans* is a major species in premenopausal, pregnant, and acute VVC women (Farr et al., 2021). VVC is one of the most common infectious vaginitis, second only to BV, and it is

estimated that about 75% of women have been infected at least once in their lifetime. In addition, recurrent VVC can affect nearly 8% of women in the world (Sobel, 2016; Matheson and Mazza, 2017; Denning et al., 2018). Many experiments have shown that vaginal microbiota changes, including species and metabolites, occur during VVC infection.

Some experiments showed no significant change in the vaginal microbiota during VVC infection. As early as 2009, studies showed that no new bacteria were found in women who frequently suffered from VVC. This study evaluated the vaginal microbial species composition of 42 women with and without frequent VVC, and evaluated microbial community diversity on this basis. The results showed no significant differences in the vaginal microbiome between the two groups. This study also showed that the vaginal flora of most women in the two groups was dominated by *Lactobacillus*, which was similar to the vaginal microbiota of most healthy women mentioned above. Therefore, it failed to provide evidence to prove the change or unusual presence of the vaginal bacterial communities in women with frequent VVC compared to those without frequent VVC (Zhou et al., 2009).

However, there are still many studies to support the theory that the vaginal microbiome of VVC patients is different from that of the normal population (Figure 1). In 1980, researchers studied *Candida albicans* in the vaginal microbiome. The results obtained from 340 vaginal specimens showed in the absence of *Candida albicans*, all microbiome increased, especially Gram-negative bacteria (Auger and Joly, 1980). Liu et al. measured the vaginal microbial communities in patients with BV and VVC. The results showed that VVC patients had high changes in their vaginal microbiome (Liu et al., 2013). The healthy vaginal microbiome is dominated by *Lactobacillus crispatus*, however, when turning from health to Chlamydia trachomatis infection (CT), VVC, and BV, *Lactobacillus crispatus* is gradually replaced by *Lactobacillus iners*. Studies have shown that CT, VVC and BV are mainly characterized by *Gardnerella, Prevotella, Megasphaera, Roseburia*, and *Atopobium*. At the same time, changes in bacterial community during genital infection will lead to significant changes in the composition of vaginal metabolites. The production of lactic acid is highly conserved in the vaginal microorganisms among different women, while the decline of lactic acid is a common sign of all the above pathological conditions (Ceccarani et al., 2019).

The microbiome of many VVC patients receiving treatment is similar to the abnormal vaginal microbiome of healthy women, suggesting that the abnormal vaginal microbiome may constitute a transitional state between disease and health, especially since many women have asymptomatic infections with *Candida* (Liu et al., 2013). Therefore, in addition to focusing on women with abnormal vaginal microbiome during VVC, we should also pay attention to women with asymptomatic infections with *Candida*, and women who appear healthy but have abnormal vaginal microbiome.

Lactobacillus treatment and defense against VVC

Over the past few years, the investigation of the vaginal microbiome has grown exponentially. These studies together found that a dominant microflora was observed in healthy vaginas: *Lactobacillus*. Therefore, it is proposed that vaginal colonization of *Lactobacillus* can reduce the risk of VVC.

It has been proved that the bacterial microbiome in the mucosal layer can achieve defense function through acidic pH regulation, release antifungal peptides and physiological control of ecological disorders. The important role of bacterial microorganisms, especially *Lactobacillus*, in maintaining vaginal health can promote their

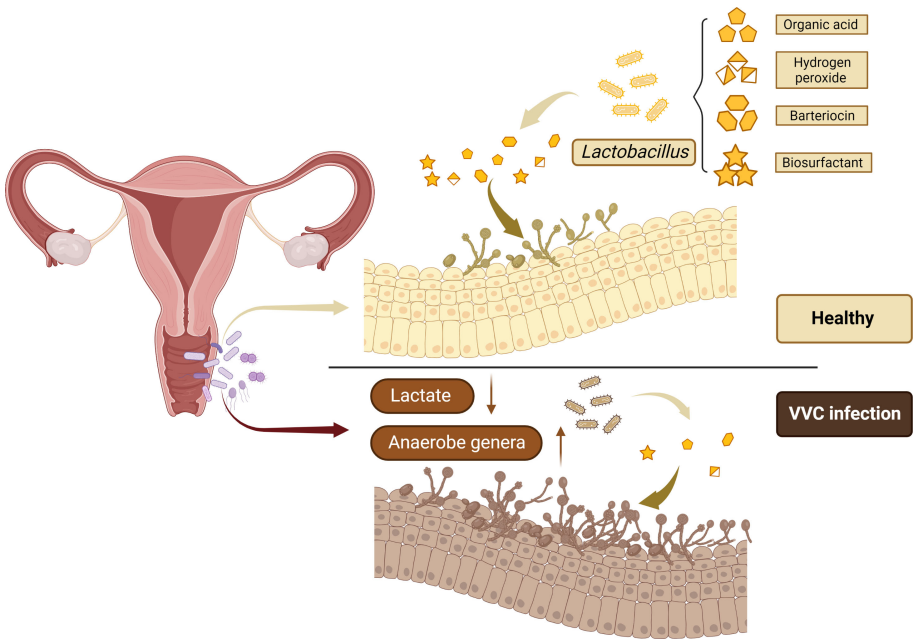

FIGURE 1
Lactobacillus is the most frequently isolated microorganism from the healthy women vagina to prevent the colonization and overgrowth of pathogens by excreting metabolic by-products, including organic acid, hydrogen peroxide, bacteriocin, and biosurfactant.

application as potential therapeutic methods for VVC, and alleviate the symptoms of VVC (Balakrishnan et al., 2022). *Lactobacillus* are thought to prevent the colonization and overgrowth of pathogens by excreting metabolic by-products and acidification of the vaginal microenvironment, helping maintain body balance. Metabolites of *Lactobacillus*, including organic acid, hydrogen peroxide, bacteriocin and biosurfactant, can contribute to antifungal effect (Wang et al., 2017; Fuochi et al., 2019). Researchers are also exploring whether *Lactobacillus* can prevent pathogens from colonizing the body, and whether it can be used to treat VVC in women. In recent years, several studies on the influence of *Lactobacillus* on VVC have drawn different conclusions, mainly regarding the role of *Lactobacillus* in preventing *Candida* colonization and treating VVC.

In vitro experiments have shown that some *Lactobacillus* strains can inhibit the adhesion and growth of *Candida albicans*, which will provide new insights into the prevention and treatment of VVC (Table 1). As early as 1999, it was reported that *Lactobacillus pentosus TV35b*, isolated from the vaginal posterior fornix secretion of prenatal patients, produced a bacteriocin like peptide to inhibit *Candida albicans* (Okkers et al., 1999). In 2001, Osset et al. found that 8 of the 15 *Lactobacillus* significantly blocked the adhesion of *Candida albicans* Y18 to vaginal cells. In liquid assays, some *Lactobacillus* had a certain degree of inhibition to *Candida albicans* Y17 (Osset et al., 2001). In 2005, Strus et al. found that *Lactobacillus delhalis* produced a large amount of H_2O_2, which more strongly inhibited *Candida albicans* growth faster than other strains isolated from healthy women vaginas, and *Lactobacillus plantarum* without H_2O_2 showed the longest inhibitory activity after 24 h (Strus et al., 2005). In 2016, researchers evaluated the *in vitro* probiotic potential of 23 *Lactobacillus* isolated from the vaginal ecosystem of healthy women for BV and VVC treatment. *In vitro* experiments have shown that all these strains had excellent adhesion properties, which can aggregate with *Gardnerella vaginalis* and *Candida albicans*, producing a large amount of hydrogen peroxide and lactic acid. These results suggested that these strains have promising probiotic potential for the prevention and treatment of BV and VVC (Santos et al., 2016). In 2017, Wang et al. found that *Lactobacillus crispatus* showed significant antimicrobial activity. Seven kinds of cell-free supernatants from the *Lactobacillus crispatus* reduced the growth of *Candida albicans*. It was shown that *Lactobacillus crispatus* is a dominant *Lactobacillus* genus, which is associated with a healthy vagina and strongly inhibits the growth and hyphal formation of *Candida albicans* (Wang et al., 2017). In 2019, Li et al. investigated the therapeutic effects and mechanisms of *Lactobacillus crispatus* and *Lactobacillus delbrueckii* on VVC caused by *Candida albicans* in a Sprague-Dawley rat model. *In vitro* results demonstrated that two *Lactobacillus* strains showed inhibitory activity against *Candida* colony forming unit counts, indicating that *Lactobacillus crispatus* and *Lactobacillus delbrueckii* may become potential adjuvants of VVC, especially in patients with antifungal drug resistance, adverse reactions or contraindications (Li et al., 2019). Some *Lactobacillus* species can produce small molecules under laboratory conditions that can block *Candida albicans* yeast-to-filament transition, which is an important virulence trait. In 2021, relevant results showed that the 1-acetyl-β-carboline produced by *Lactobacillus* can prevent the yeast-to-filament transition of *Candida albicans* by inhibiting Yak1 (MacAlpine et al., 2021). Researchers examined the inhibitory activity of bacteriocin-like inhibitory substances (BLISs) from *Lactobacillus* and *Streptococci* on *Candida albicans* and non-*Candida albicans* isolated from patients with VVC. Using agar pore diffusion test, BLISs can inhibit both *Candida albicans* and non-*Candida albicans* (Hefzy et al., 2021). The bactericidal effect of *Lactobacillus casei* on the main VVC pathogenic species *Candida albicans, Candida tropicalis, Clostridium novigen and Paracandida* was investigated by calculating the colony forming units after co-cultivation. *Lactobacillus casei* had an inhibitory effect on all tested *Candida* genera, and *Lactobacillus casei* could reduce the formation of *Candida albicans* mycelia and early biofilm, showing a strong anti-*Candida* effect (Paniagua et al., 2021).

TABLE 1 *In vitro* experiments.

Authors	Time	Results	References
Okkers et al.	1999	*Lactobacillus pentosus TV35b* produced a bacteriocin like peptide to inhibit *Candida albicans*	(Okkers et al., 1999)
Osset et al.	2001	Some *Lactobacillus* had a certain degree of inhibition to *Candida albicans* Y17.	(Osset et al., 2001)
Strus et al.	2005	*Lactobacillus delhalis* produced a large amount of H2O2 and inhibited *Candida albicans* growth. *Lactobacillus plantarum* without H_2O_2 showed the longest inhibitory activity after 24 h	(Strus et al., 2005)
Santos et al.	2016	*Lactobacillus* strains had excellent adhesion properties, which can aggregate with *Gardnerella vaginalis* and *Candida albicans*, producing a large amount of hydrogen peroxide and lactic acid.	(Santos et al., 2016)
Wang et al.	2017	7 kinds of cell-free supernatants from the *Lactobacillus crispatus* reduced the growth of *Candida albicans*.	(Wang et al., 2017)
Li et al.	2019	*Lactobacillus* strains showed inhibitory activity against *Candida* colony forming unit counts, indicating that *Lactobacillus crispatus* and *Lactobacillus delbrueckii* may become potential adjuvants of VVC.	(Li et al., 2019)
MacAlpine et al.	2021	1-acetyl-β-carboline produced by *Lactobacillus* can prevent the yeast-to-filament transition of *Candida albicans* by inhibiting Yak1.	(MacAlpine et al., 2021)
Hefzy et al.	2021	Bacteriocin-like inhibitory substances can inhibit both *Candida albicans* and non-*Candida albicans*.	(Hefzy et al., 2021)
Paniagua et al.	2021	*Lactobacillus casei* had an inhibitory effect on all tested *Candida* genera, and *Lactobacillus casei* could reduce the formation of *Candida albicans* mycelia and early biofilm.	(Paniagua et al., 2021)

Lactobacillus strains and their products can inhibit the growth of *Candida*, and clinical studies are also in progress (Table 2). In 2001, a trial of 64 healthy women was conducted in which oral capsules of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 were taken daily. The results showed that the combination of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 can not only be safely used for daily use in healthy women, but also reduce the potential pathogenic bacteria and yeast colonization in vagina (Reid et al., 2003). De Seta et al. also evaluated the effect of the application of *Lactobacillus plantarum* P17630 on the recovery of vaginal microbiota and prevention of recurrence in women with acute VVC. These results confirmed the role of *Lactobacillus plantarum* P17630 as a potential empirical preventive agent, which can reduce vaginal discomfort after routine treatment of acute VVC and improve

TABLE 2 Clinical studies.

Authors	Time	Results	References
Reid et al.	2001	The combination of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 can not only be used for daily use in healthy women, but also reduce the potential pathogenic bacteria and yeast colonization in vagina.	(Reid et al., 2003)
De Seta et al.	2014	*Lactobacillus plantarum* P17630 can reduce vaginal discomfort after routine treatment of acute VVC and improve vaginal pH.	(De Seta et al., 2014)
Russo et al.	2019	The combination of a *Lactobacillus* mixture with lactoferrin is a safe and effective auxiliary method to reduce VVC symptoms and recurrence.	(Russo et al., 2019)
Oerlemans et al.	2020	*Lactobacillus* can be used for VVC treatment in the future.	(Oerlemans et al., 2020)
De Gregorio et al.	2020	Biosurfactants from *Lactobacillus crispatus* BC1 can interfere with the adherence of *Candida in vivo* and *in vitro*, indicating its potential as a preventive measure against *Candida* mucosal damage during VVC.	(De Gregorio et al., 2020)

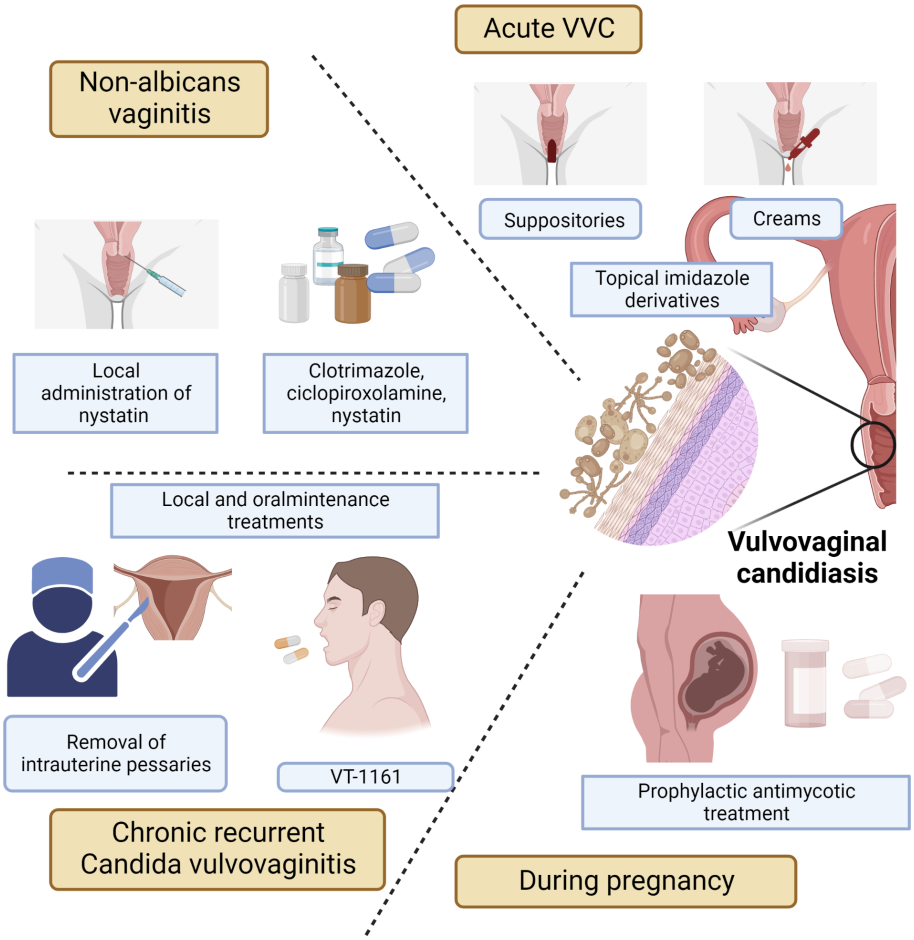

FIGURE 2
Treatments for acute VVC or recurrent VVC, including prescription oral formulations, over-the-counter topical preparations, and vaginal suppositories.

vaginal pH (De Seta et al., 2014). In 2019, Russo et al. found that the combination of a *Lactobacillus* mixture with lactoferrin is a safe and effective auxiliary method to reduce VVC symptoms and recurrence (Russo et al., 2019). Based on *in vitro* evaluation, Oerlemans et al. selected three strains from the *Lactobacillus* genus complex (*Lactobacillus rhamnosus GG, Lactobacillus pentosus KCA1*, and *Lactobacillus plantarum WCFS1*) and prepared them with gel for vaginal use. The gel was evaluated in 20 patients suffering from acute VVC, whose fungal concentrations were similar to those of women treated with fluconazole. These results pointed out the important aspects of choosing *Lactobacillus* for VVC treatment in the future (Oerlemans et al., 2020). Besides, the safety and antimicrobial activity of biosurfactants (BS) isolated from *Lactobacillus* vaginalis strains on *Candida* genus were studied. The results showed that BS from *Lactobacillus crispatus* BC1 can interfere with the adherence of *Candida in vivo* and *in vitro*, indicating its potential as a preventive measure against *Candida* mucosal damage during VVC (De Gregorio et al., 2020).

The above experiments focused on the possibility of *Lactobacillus* to prevent VVC. In view of the great potential of *Lactobacillus*, whether other probiotics can be used for the treatment and defense of VVC deserves further research. In addition, it has been shown that certain biological components or subcomponents can inhibit the growth of *Candida*. de Freitas et al. used F2 and sub-fraction F2.4 tannins from Stryphnodendron adstringens stem bark to treat mice with vaginal infection with *Candida albicans* and analyzed vaginal histopathology and fungal load. The results showed that F2 and F2.4 have efficacy in controlling candidiasis in mouse models (de Freitas et al., 2018).

It should also be noted that there exist differences in the vaginal microbiome among women of different races, such as the body's own immune system, the different amounts and composition of vaginal secretions. Therefore, it needs to be further studied whether treatment and defense of *Lactobacillus* and other microbiota against VVC are also related to the host's genetic factors, behavior, and cultural differences (Denning et al., 2018).

Conclusion

VVC is a complex disease, and its symptoms are affected by host physiology, fungal biology and immune response. Currently, there are many treatments for VVC, mainly including prescription oral dosage forms, over-the-counter topical preparations, and vaginal suppositories (Figure 2).

Given the projected global prevalence and economic burden of VVC in the next decade, high-income countries need better solutions and improved quality of care for affected women. With the increasing awareness of human microorganisms, probiotic treatment has been a hot topic in recent years. In many *in vivo* and *in vitro* experiments, *Lactobacillus* have been shown to have a certain effect on the prevention and treatment of VVC, but clinical data are still scarce and need to be further explored. It is believed that with the advancement of technology, the composition of vaginal microbiome and the preventive and therapeutic effects of vaginal microbiome on VVC can be further elucidated.

Author contributions

ZS, XG and BQ had the idea for the article. ZX and CJ performed the literature search and data analysis. JW and YL drafted and critically revised the work. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Amabebe, E., and Anumba, D. O. C. (2018). The vaginal microenvironment: The physiologic role of lactobacilli. *Front. Med. (Lausanne)* 5. doi: 10.3389/fmed.2018.00181

Auger, P., and Joly, J. (1980). Microbial flora associated with candida albicans vulvovaginitis. *Obstetrics Gynecol.* 55 (3), 397–401. doi: 10.1097/00006250-198003000-00029

Balakrishnan, S. N., Yamang, H., Lorenz, M. C., Chew, S. Y., and Than, L. T. L. (2022). Role of vaginal mucosa, host immunity and microbiota in vulvovaginal candidiasis. *Pathogens* 11 (6), 618. doi: 10.3390/pathogens11060618

Calonghi, N., Parolin, C., Sartor, G., Verardi, L., Giordani, B., Frisco, G., et al. (2017). Interaction of vaginal lactobacillus strains with HeLa cells plasma membrane. *Benef Microbes* 8 (4), 625–633. doi: 10.3920/BM2016.0212

Ceccarani, C., Foschi, C., Parolin, C., D'Antuono, A., Gaspari, V., Consolandi, C., et al. (2019). Diversity of vaginal microbiome and metabolome during genital infections. *Sci. Rep.* 9 (1), 14095. doi: 10.1038/s41598-019-50410-x

Champer, M., Wong, A. M., Champer, J., Brito, I. L., Messer, P. W., Hou, J. Y., et al. (2018). The role of the vaginal microbiome in gynaecological cancer. *BJOG* 125 (3), 309–315. doi: 10.1111/1471-0528.14631

Chee, W. J. Y., Chew, S. Y., and Than, L. T. L. (2020). Vaginal microbiota and the potential of lactobacillus derivatives in maintaining vaginal health. *Microb. Cell Fact* 19 (1), 203. doi: 10.1186/s12934-020-01464-4

Chen, X., Lu, Y., Chen, T., and Li, R. (2021). The female vaginal microbiome in health and bacterial vaginosis. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.631972

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., et al. (2017). The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat. Commun.* 8 (1), 875. doi: 10.1038/s41467-017-00901-0

Culhane, J. F., Rauh, V., McCollum, K. F., Elo, I. T., and Hogan, V. (2002). Exposure to chronic stress and ethnic differences in rates of bacterial vaginosis among pregnant women. *Am. J. Obstet Gynecol* 187 (5), 1272–1276. doi: 10.1067/mob.2002.127311

de Freitas, A. L. D., Kaplum, V., Rossi, D. C. P., da Silva, L. B. R., Melhem, M. S. C., Taborda, C. P., et al. (2018). Proanthocyanidin polymeric tannins from stryphnodendron adstringens are effective against candida spp. isolates and for vaginal candidiasis treatment. *J. Ethnopharmacol.* 216, 184–190. doi: 10.1016/j.jep.2018.01.008

De Gregorio, P. R., Parolin, C., Abruzzo, A., Luppi, B., Protti, M., Mercolini, L., et al. (2020). Biosurfactant from vaginal lactobacillus crispatus BC1 as a promising agent to interfere with candida adhesion. *Microb. Cell Fact* 19 (1), 133. doi: 10.1186/s12934-020-01390-5

Denning, D. W., Kneale, M., Sobel, J. D., and Rautemaa-Richardson, R. (2018). Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect. Dis.* 18 (11), e339–e347. doi: 10.1016/S1473-3099(18)30103-8

De Seta, F., Parazzini, F., De Leo, R., Banco, R., Maso, G. P., De Santo, D., et al. (2014). Lactobacillus plantarum P17630 for preventing candida vaginitis recurrence: a retrospective comparative study. *Eur. J. Obstet Gynecol Reprod. Biol.* 182, 136–139. doi: 10.1016/j.ejogrb.2014.09.018

Di Bella, J. M., Bao, Y., Gloor, G. B., Burton, J. P., and Reid, G. (2013). High throughput sequencing methods and analysis for microbiome research. *J. Microbiol. Methods* 95 (3), 401–414. doi: 10.1016/j.mimet.2013.08.011

Farr, A., Effendy, I., Frey Tirri, B., Hof, H., Mayser, P., Petricevic, L., et al. (2021). Guideline: Vulvovaginal candidosis (AWMF 015/072, level S2k). *Mycoses* 64 (6), 583–602. doi: 10.1111/myc.13248

Fuochi, V., Cardile, V., Petronio Petronio, G., and Furneri, P. M. (2019). Biological properties and production of bacteriocins-like-inhibitory substances by lactobacillus sp. strains from human vagina. *J. Appl. Microbiol.* 126 (5), 1541–1550. doi: 10.1111/jam.14164

Gow, N. A., and Hube, B. (2012). Importance of the candida albicans cell wall during commensalism and infection. *Curr. Opin. Microbiol.* 15 (4), 406–412. doi: 10.1016/j.mib.2012.04.005

Gupta, V. K., Paul, S., and Dutta, C. (2017). Geography, ethnicity or subsistence-specific variations in human microbiome composition and diversity. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.01162

Hall, R. A., and Noverr, M. C. (2017). Fungal interactions with the human host: exploring the spectrum of symbiosis. *Curr. Opin. Microbiol.* 40, 58–64. doi: 10.1016/j.mib.2017.10.020

Hefzy, E. M., Khalil, M. A. F., Amin, A. A. I., Ashour, H. M., and Abdelaliem, Y. F. (2021). Bacteriocin-like inhibitory substances from probiotics as therapeutic agents for candida vulvovaginitis. *Antibiotics (Basel)* 10 (3), 306. doi: 10.3390/antibiotics10030306

Jayaram, P. M., Mohan, M. K., and Konje, J. (2020). Bacterial vaginosis in pregnancy - a storm in the cup of tea. *Eur. J. Obstet Gynecol Reprod. Biol.* 253, 220–224. doi: 10.1016/j.ejogrb.2020.08.009

Kalia, N., Singh, J., and Kaur, M. (2020). Microbiota in vaginal health and pathogenesis of recurrent vulvovaginal infections: a critical review. *Ann. Clin. Microbiol. Antimicrob.* 19 (1), 5. doi: 10.1186/s12941-020-0347-4

Khanna, V., Lakshmi, K., Khanna, R., Verma, S., and Acharya, V. (2022). Intestinal parasitic infections among diabetic patients in tertiary care hospital. *Advanced Gut Microbiome Res.* 2022. doi: 10.1155/2022/4829943

Li, T., Liu, Z., Zhang, X., Chen, X., and Wang, S. (2019). Local probiotic lactobacillus crispatus and lactobacillus delbrueckii exhibit strong antifungal effects against vulvovaginal candidiasis in a rat model. *Front. Microbiol.* 10, 1033. doi: 10.3389/fmicb.2019.01033

Liu, M. B., Xu, S. R., He, Y., Deng, G. H., Sheng, H. F., Huang, X. M., et al. (2013). Diverse vaginal microbiomes in reproductive-age women with vulvovaginal candidiasis. *PloS One* 8 (11), e79812. doi: 10.1371/journal.pone.0079812

Lopez-Garcia, P., and Moreira, D. (2020). The syntrophy hypothesis for the origin of eukaryotes revisited. *Nat. Microbiol.* 5 (5), 655–667. doi: 10.1038/s41564-020-0710-4

MacAlpine, J., Daniel-Ivad, M., Liu, Z., Yano, J., Revie, N. M., Todd, R. T., et al. (2021). A small molecule produced by lactobacillus species blocks candida albicans filamentation by inhibiting a DYRK1-family kinase. *Nat. Commun.* 12 (1), 6151. doi: 10.1038/s41467-021-26390-w

Ma, B., Forney, L. J., and Ravel, J. (2012). Vaginal microbiome: rethinking health and disease. *Annu. Rev. Microbiol.* 66, 371–389. doi: 10.1146/annurev-micro-092611-150157

Matheson, A., and Mazza, D. (2017). Recurrent vulvovaginal candidiasis: A review of guideline recommendations. *Aust. N Z J. Obstet Gynaecol.* 57 (2), 139–145. doi: 10.1111/ajo.12592

Mojzsis, S. J., Arrhenius, G., McKeegan, K. D., Harrison, T. M., Nutman, A. P., and Friend, C. R. (1996). Evidence for life on earth before 3,800 million years ago. *Nature* 384 (6604), 55–59. doi: 10.1038/384055a0

Noyes, N., Cho, K. C., Ravel, J., Forney, L. J., and Abdo, Z. (2018). Associations between sexual habits, menstrual hygiene practices, demographics and the vaginal microbiome as revealed by Bayesian network analysis. *PloS One* 13 (1), e0191625. doi: 10.1371/journal.pone.0191625

Oerlemans, E. F. M., Bellen, G., Claes, I., Henkens, T., Allonsius, C. N., Wittouck, S., et al. (2020). Impact of a lactobacilli-containing gel on vulvovaginal candidosis and the vaginal microbiome. *Sci. Rep.* 10 (1), 7976. doi: 10.1038/s41598-020-64705-x

Okkers, D. J., Dicks, L. M., Silvester, M., Joubert, J. J., and Odendaal, H. J. (1999). Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from lactobacillus pentosus with a fungistatic effect on candida albicans. *J. Appl. Microbiol.* 87 (5), 726–734. doi: 10.1046/j.1365-2672.1999.00918.x

Onderdonk, A. B., Delaney, M. L., and Fichorova, R. N. (2016). The human microbiome during bacterial vaginosis. *Clin. Microbiol. Rev.* 29 (2), 223–238. doi: 10.1128/CMR.00075-15

Osset, J., Garcia, E., Bartolome, R. M., and Andreu, A. (2001). [Role of lactobacillus as protector against vaginal candidiasis]. *Med. Clin. (Barc)* 117 (8), 285–288. doi: 10.1016/s0025-7753(01)72089-1

Pang, Q., Liu, W., Cui, F., Kan, S., and Li, X. (2022). Biased genotype distributions of candida albicans strains associated with 649 clinical vulvovaginal candidiasis in China. *Mycopathologia* 187 (5), 427–437. doi: 10.1007/s11046-022-00671-4

Paniagua, A. L., Correia, A. F., Pereira, L. C., de Alencar, B. M., Silva, F. B. A., Almeida, R. M., et al. (2021). Inhibitory effects of lactobacillus casei shirota against both candida auris and candida spp. isolates that cause vulvovaginal candidiasis and are resistant to antifungals. *BMC Complement Med. Ther.* 21 (1), 237. doi: 10.1186/s12906-021-03405-z

Parolin, C., Frisco, G., Foschis, C., Giordani, B., Salvo, M., Vitali, B., et al. (2018). Lactobacillus crispatus BC5 interferes with chlamydia trachomatis infectivity through integrin modulation in cervical cells. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.02630

Parolin, C., Marangoni, A., Laghi, L., Foschi, C., Palomino, R. A. N., Calonghi, N., et al. (2015). Isolation of vaginal lactobacilli and characterization of anti-candida activity. *PloS One* 10 (6), e0131220. doi: 10.1371/journal.pone.0131220

Paul, K., Boutain, D., Manhart, L., and Hitti, J. (2008). Racial disparity in bacterial vaginosis: the role of socioeconomic status, psychosocial stress, and neighborhood characteristics, and possible implications for preterm birth. *Soc. Sci. Med.* 67 (5), 824–833. doi: 10.1016/j.socscimed.2008.05.017

Petrova, M. I., Reid, G., Vaneechoutte, M., and Lebeer, S. (2017). Lactobacillus iners: Friend or foe? *Trends Microbiol.* 25 (3), 182–191. doi: 10.1016/j.tim.2016.11.007

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U.S.A.* 108 Suppl 1, 4680–4687. doi: 10.1073/pnas.1002611107

Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beuerman, D., Poehner, R., et al. (2003). Oral use of lactobacillus rhamnosus GR-1 and l. fermentum RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunol. Med. Microbiol.* 35 (2), 131–134. doi: 10.1016/S0928-8244(02)00465-0

Russo, R., Superti, F., Karadja, E., and De Seta, F. (2019). Randomised clinical trial in women with recurrent vulvovaginal candidiasis: Efficacy of probiotics and lactoferrin as maintenance treatment. *Mycoses* 62 (4), 328–335. doi: 10.1111/myc.12883

Santos, C. M. A., Pires, M. C. V., Leao, T. L., Hernandez, Z. P., Rodriguez, M. L., Martins, A. K. S., et al. (2016). Selection of lactobacillus strains as potential probiotics for vaginitis treatment. *Microbiol. (Reading)* 162 (7), 1195–1207. doi: 10.1099/mic.0.000302

Schwebke, J. R., Richey, C. M., and Weiss, H. L. (1999). Correlation of behaviors with microbiological changes in vaginal flora. *J. Infect. Dis.* 180 (5), 1632–1636. doi: 10.1086/315065

Sobel, J. D. (2007). Vulvovaginal candidosis. *Lancet* 369 (9577), 1961–1971. doi: 10.1016/S0140-6736(07)60917-9

Sobel, J. D. (2016). Recurrent vulvovaginal candidiasis. *Am. J. Obstet Gynecol* 214 (1), 15–21. doi: 10.1016/j.ajog.2015.06.067

Strus, M., Brzychczy-Wloch, M., Kucharska, A., Gosiewski, T., and Heczko, P. B. (2005). [Inhibitory activity of vaginal lactobacillus bacteria on yeasts causing vulvovaginal candidiasis]. *Med. Dosw Mikrobiol* 57 (1), 7–17.

Wang, S., Wang, Q., Yang, E., Yan, L., Li, T., and Zhuang, H. (2017). Antimicrobial compounds produced by vaginal lactobacillus crispatus are able to strongly inhibit candida albicans growth, hyphal formation and regulate virulence-related gene expressions. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00564

Wang, Y., Wu, J., and Cao, Y. (2015). The extended spectrum β-lactamases (ESBL) and virulence genes of intestinal enteroaggregative escherichia coli (EAEC) in healthy elderly individuals. *Int. J. Clin. Exp. Med.* 8 (11), 20953.

Witkin, S. S., and Forney, L. J. (2020). The microbiome and women's health: perspectives and controversies. *BJOG* 127 (2), 127. doi: 10.1111/1471-0528.16010

Wu, J., Guo, N., Zhang, X., Xiong, C., Liu, J., Xu, Y., et al. (2019). HEV-LFS: A novel scoring model for patients with hepatitis e virus-related liver failure. *J. Viral Hepatitis* 26 (11), 1334–1343. doi: 10.1111/jvh.13174

Wu, J., Huang, F., Ling, Z., Liu, S., Liu, J., Fan, J., et al. (2020). Altered faecal microbiota on the expression of Th cells responses in the exacerbation of patients with hepatitis e infection. *J. Viral Hepatitis* 27 (11), 1243–1252. doi: 10.1111/jvh.13344

Xiang, Z., Li, J., Lu, D., Wei, X., and Xu, X. (2022). Advances in multi-omics research on viral hepatitis. *Front. Microbiol.* 3365. doi: 10.3389/fmicb.2022.987324

Younes, J. A., Lievens, E., Hummelen, R., van der Westen, R., Reid, G., and Petrova, M. I. (2018). Women and their microbes: The unexpected friendship. *Trends Microbiol.* 26 (1), 16–32. doi: 10.1016/j.tim.2017.07.008

Zhou, X., Brown, C. J., Abdo, Z., Davis, C. C., Hansmann, M. A., Joyce, P., et al. (2007). Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME J.* 1 (2), 121–133. doi: 10.1038/ismej.2007.12

Zhou, X., Hansmann, M. A., Davis, C. C., Suzuki, H., Brown, C. J., Schutte, U., et al. (2010). The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. *FEMS Immunol. Med. Microbiol.* 58 (2), 169–181. doi: 10.1111/j.1574-695X.2009.00618.x

Zhou, X., Westman, R., Hickey, R., Hansmann, M. A., Kennedy, C., Osborn, T. W., et al. (2009). Vaginal microbiota of women with frequent vulvovaginal candidiasis. *Infect. Immun.* 77 (9), 4130–4135. doi: 10.1128/IAI.00436-09

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Mingzhu Li,
Peking University People's Hospital, China
Dai Zhang,
First Hospital, Peking University, China
Qinping Liao,
Tsinghua University, China

*CORRESPONDENCE
Aiping Fan
fanaipinger@163.com
Fengxia Xue
xuefengxia@tmu.edu.cn

†These authors have contributed equally to this work

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Analysis of the related factors of atypical squamous cells of undetermined significance (ASC-US) in cervical cytology of post-menopausal women

Bijun Li[1,2†], Lichang Dong[1,2†], Chen Wang[1,2], Jia Li[1,2], Xue Zhao[1,2], Mengting Dong[1,2], Huanrong Li[1,2], Xiaotong Ma[1,2], Yalan Dong[1,2], Ming Wu[1,2], Ye Yan[1,2], Aiping Fan[1,2*] and Fengxia Xue[1,2*]

[1]Department of Gynecology and Obstetrics, Tianjin Medical University General Hospital, Tianjin, China, [2]Tianjin Key Laboratory of Female Reproductive Health and Eugenic, Department of Gynecology and Obstetrics, Tianjin Medical University General Hospital, Tianjin, China

Introduction: Atrophy of the reproductive tract mucosa caused by the decrease of estrogen may increase the detection rate of ASC-US in cervical cytology of post-menopausal women. In addition, other pathogenic infections and inflammation can change the cellular morphology and increase the detection rate of ASC-US. However, further studies are needed to elucidate whether the high detection rate of ASC-US in post-menopausal women leads to the high referral rate of colposcopy.

Methods: This retrospective study was conducted to document ASC-US in cervical cytology reports at the Department of Cytology at Gynecology and Obstetrics, Tianjin Medical University General Hospital between January 2006 and February 2021. We then analyzed 2,462 reports of women with ASC-US at the Cervical Lesions Department. A total of 499 patients with ASC-US and 151 cytology with NILM participants underwent vaginal microecology tests.

Results: The average reporting rate of ASC-US in cytology was 5.7%. The detection rate of ASC-US in women aged > 50 years (7.0%) was significantly higher than that in women aged ≤50 years (5.0%) (P<0.05). The CIN2+ detection rate was significantly lower in the post- (12.6%) than in pre-menopausal (20.5%) patients with ASC-US (P <0.05). The prevalence of abnormal reporting rate of vaginal microecology was significantly lower in the pre-menopausal group (56.2%) than that in the post-menopausal group (82.9%) (P<0.05). The prevalence of bacterial vaginosis (BV) (19.60%) was relatively high in the pre-menopausal group, but the abundance of bacteria-inhibiting flora (40.79%) was mainly an abnormality in the post-menopausal group. The vaginal microecological abnormality rate of the women with HR-HPV (-) of ASC-US was 66.22%, which was significantly higher than that of the HR-HPV (-) and the NILM group (52.32%; P<0.05).

Discussion: The detection rate of ASC-US in women aged > 50 years was higher than that ≤50 years, but the detection rate of CIN2+ was lower in the post-menopausal women with ASC-US. However, vaginal microecological abnormalities may increase the false-positive diagnosis rate of ASC-US. The

vaginal microecological abnormalities of the menopausal women with ASC-US are mainly attributed to infectious diseases such as BV, and it mainly occurs in the post-menopausal women was bacteria-inhibiting flora. Therefore, to avoid the high referral rate for colposcopy, more attention should be paid to the detection of vaginal microecology.

KEYWORDS

cervical cytology, post-menopausal women, atypical squamous cells, cervical intraepithelial neoplasias, vaginal microecology

1 Introduction

Cervical cancer remains a significant public health concern among women in China. The National Cancer Center (NCC) of China reported in the 2016 nationwide statistics for cancer that the incidence and mortality rates of cervical cancer were ranked the fifth and seventh among all the female malignancies, respectively, and these rates continue to be on the rise (Zheng et al., 2022). Therefore, selecting more effective screening methods and improving the diagnosis rate of cervical precancerous lesions are of great significance in accelerating the elimination of cervical cancer. Atypical squamous cells (ASC) are the most prevalent form of all the abnormal cervical cytology interpretations (Nayar and Wilbur, 2015). However, the management of women with ASC-US remains a clinical challenge because of the variable underlying processes from human papillomavirus (HPV)-unrelated, non-neoplastic conditions to HPV-related cervical intraepithelial neoplasia (CIN) and carcinomas (Solomon et al., 2002). ASC-US is equivocal and the diagnosis is highly subjective. In addition to HPV infection and tumor-related factors, other pathogenic infections, inflammation, atrophy, intrauterine device (IUD), and air dryness can change the cellular morphology and may lead to the misdiagnosis of ASC-US (Watson et al., 2015; Bruno et al., 2019; Koh et al., 2019).Continuous infection with high-risk human papillomavirus (HR-HPV) is necessary for the development of cervical cancer, which is clearly associated with abnormal cytology. In recent years, many countries, including the United States, have recommended triage HPV testing after cytology showing ASC-US and colposcopy for all HR-HPV-positive cases (Zeferino et al., 2018; Kyrgiou et al., 2020; Perkins et al., 2020; Bhatla et al., 2020). These strategies may be associated with excessive care (Zeferino et al., 2018; Kyrgiou et al., 2020; Perkins et al., 2020; Bhatla et al., 2020). First, the distribution of HR-HPV varies geographically across continents, particularly HPV52 and HPV58, whose prevalences are notably higher in cases of invasive cervical cancer in China than those of HPV45 and HPV18 (Zhou et al., 2018). Second, based on the different carcinogenicities of HPV genotypes, the HPV genotype has various impacts on the development of cervical cancer and CIN. It has been reported that women with ASC-US and HPV16 or HPV18 positive are more likely to develop CIN3+ than women with other HR HPV types (Stoler et al., 2011). It has also been reported that women with ASC-US with HPV16, 18, 31, 33, or 58 genotypes are most likely to develop CIN2 (Wang et al., 2021). As a result, type-specific HPV identification appears to be a reasonable strategy for reducing the colposcopy burden in women with ASC-US and improving the risk stratification. In addition, the transformation zone of post-menopausal women moved up, squamous metaplasia was slow, immunity declined, and the lower reproductive tract was more susceptible to carcinogenic factors, which facilitated the development of persistent infection (Brotman et al., 2014). Studies have shown that the rate of cytological abnormalities in the women over 50 years of age is dramatically higher than that in the women aged < 50 (Zheng et al., 2019; Wang et al., 2020b; Tao et al., 2021). However, it is still debatable whether there is a difference in the prognosis between pre- and post-menopausal women. It has been reported that the detection rate of CIN2+ in women with ASC-US over 50 years of age is much higher than that under 50 years of age (Wang et al., 2020b). In one survey, the incidence of CIN3+ lesions was higher in women younger than 30 years and those older than 60 years old (Tai et al., 2018). However, some studies have suggested that there is no difference in the HSIL+ detection rate in women with ASCUS before and after 50 years old (Abdulaziz et al., 2020). Therefore, the relationship between ASC-US and histopathology in post-menopausal women remains unclear and requires further investigation.

In addition to HPV infection, other synergistic factors also affect the cervical cytology results. Bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), trichomoniasis and aerobic vaginitis (AV) are associated with an increased risk of CIN (Peres et al., 2015; Plisko et al., 2021). Liu et al. reported that the positive rate of HR-HPV increased with the cytological severity, but vaginal microecological abnormalities were mainly related to ASC-US and LSIL (Liu et al., 2021). Some other studies have also revealed that vaginal microecological abnormalities are associated with ASC-US (Gomes de Oliveira et al., 2018). In addition, it has been reported that post-menopausal women are prone to microecological disorders, such as bacterial community disorder and impaired vaginal immune barrier due to decreased estrogen levels, atrophy and thinning of vaginal mucosa, decreased glycogen content in epithelial cells, and the decrease in the species of *Lactobacillus* (Xu et al., 2021). It has also been confirmed that the "atrophic" form caused by the decreased hormone levels can increase the detection rate of ASC-US in post-menopausal women (Cakmak and Köseoğlu, 2014).

In the current study, we document and analyze the reporting rates of the age-stratified ASC-US, the immediate histopathology of ASC-US along with HR-HPV genotype distribution, outcomes of post-menopausal women with ASC-US, and the correlation between

vaginal microecology and ASC-US from the department of Gynecology and Obstetrics, Tianjin Medical University General Hospital. This aims at offering new parameters to design an optimal strategy for the triage of pre- and post-menopausal patients with ASC-US.

2 Materials and methods

2.1 Study population

This retrospective study was conducted to document the cervical cytology reports of patients with ASC-US at the Department of Cytology at Gynecology and Obstetrics, Tianjin Medical University General Hospital (TMUGH) between January 2006 and February 2021. We then analyzed 2,462 reports of women with ASC-US who underwent HPV genotyping, colposcopy, and histopathological examination at the Cervical Lesions Department of Obstetrics and Gynecology between January 2010 and December 2021. It is important to emphasize that a total of 499 participants with ASC-US and 151 with HR-HPV-negative and NILM cytology underwent vaginal microecology tests. The inclusion and exclusion criteria are shown in Figure 1. 2462 patients underwent colposcopy and biopsy. The menstrual status data were available for 2,018 women, while the information on the menstrual status was unknown for 444 women. Of the 2,018 women, 507 were confirmed as post-menopausal. The mean age of the post-menopausal women was 50.23 years. Using the previously reported age cut-off recommendations to classify menopause (Holt et al., 2017), the mean age of our study was then used as a cut-off to define the post-menopausal status in the remaining 444 women. With this classification, an additional 197 women were included in the post-menopausal group, resulting in a total number of 707 women in the studied menopausal group and 1,755 in the pre-menopausal group.

2.2 Cytology testing

The Liquid-based cytological test (LCT) method was used in this study. LCT preparation was performed by Becton, Dickinson and Company. Cytological evaluation was performed by two cytopathologists at the Department of Cytology at Gynecology and Obstetrics, TMUGH. Cytological results were classified using the 2001 TBS criteria for reporting cervical cytology before 2014 (Solomon et al., 2002), and the rest were classified using the 2014 TBS criteria (Nayar and Wilbur, 2015). A cytopathologist who was blinded to the results reviewed the cases with an ASC-US interpretation or worse.

2.3 HPV genotyping

The HR-HPV genotypes (16,18,31,33,35,39,45,51,52,53,56, 58,59,66,68,73,82 and 83) were detected using a polymerase chain reaction-reverse dot blot (PCR-RDB) HPV genotyping kit (YaNeng Biosciences, Shenzhen, China). The procedures were performed in accordance with the manufacturer's instructions. A small number of patients were tested for HPV in other hospitals, and the HPV test was performed using the Roche Cobas 4800 HPV DNA and HC2 tests. The Roche Cobas 4800 HPV DNA test can detect 14 HR-HPV types, including HPV16,18,31,33,35,39,45,51,52,56,58,59,66 and 68. HC2 test can be used for the quantitative detection of 13 high-risk HPV types, without HPV genotype, and ≥1.0pg/mL is considered positive.

2.4 Colposcopy and histological examination

ASC-US with positive HR-HPV or negative HR-HPV and more than twice ASC-US cytology were referred for colposcopy for biopsy. Colposcopy and biopsy were performed by experienced gynecological specialists from TMUGH. The duration between cytological sampling and colposcopy did not exceed 60 days. During the colposcopy, all the visually abnormal areas were biopsied. Quadrants with normal colposcopic impressions were biopsied at the squamocolumnar junction ("random biopsy"). Endocervical curettage(ECC)is performed when the TZ is not visible or fully visible. All histological slides were reviewed by two gynecological pathologists who were blinded to the cytology results at TMUGH. Immunohistochemistry was used to adjudicate difficult or equivocal diagnoses.

2.5 Vaginal microecology analysis

Women with suspicious vaginitis, uncomfortable symptoms, or routine physical examination underwent vaginal microecological analysis before and after the cytology test. An unlubricated sterile speculum was inserted before any other vaginal examinations were performed. Sterile long cotton swabs were used to obtain the vaginal discharge from the upper lateral vaginal wall for vaginal pH measurements (special indicator paper, pH 3.8–5.4; Shanghai SSS Reagent Co. Ltd., Shanghai, China) and microscopic examinations. Vaginal discharge smears were spread on 3 glass slides for immediate microscopic examination. AV, BV, Trichomoniasis, and VVC were defined according to their recognized standards. The Nugent score was used to diagnose BV and was calculated by assessing the number of *Lactobacillus* morphotypes (scored as 0–4), *G. vaginalis* morphotypes (scored as 0–4), and *Mobiluncus* morphotypes (scored as 0–2). A Nugent score of 7–10 was interpreted as consistent with BV, a score of 4–6 as intermediate (BV intermediate type), and a score of 0–3 as negative for BV. Wet-mount microscopy has traditionally been used as the preferred diagnostic test for trichomoniasis in women. A diagnosis of Candida vaginitis is indicated by a wet preparation (saline, 10% KOH) of vaginal discharge demonstrating budding yeasts, hyphae, or pseudohyphae (Workowski et al., 2021). The diagnosis of aerobic vaginitis (Donders et al., 2017) by microscopic examination included the evaluation of lactobacillary grades (LBG) (scored as 0–2), the number of leukocytes (scored as 0–2), the proportion of toxic leukocytes (scored as 0–2), the type of background bacteria (score 0–2), and the number of parabasal epithelial cells under the wet film using a phase-contrast microscope (×400, score 0–2). An AV score ≥3 indicated AV. Two or more types of pathogenic microorganisms coexisting in the vagina

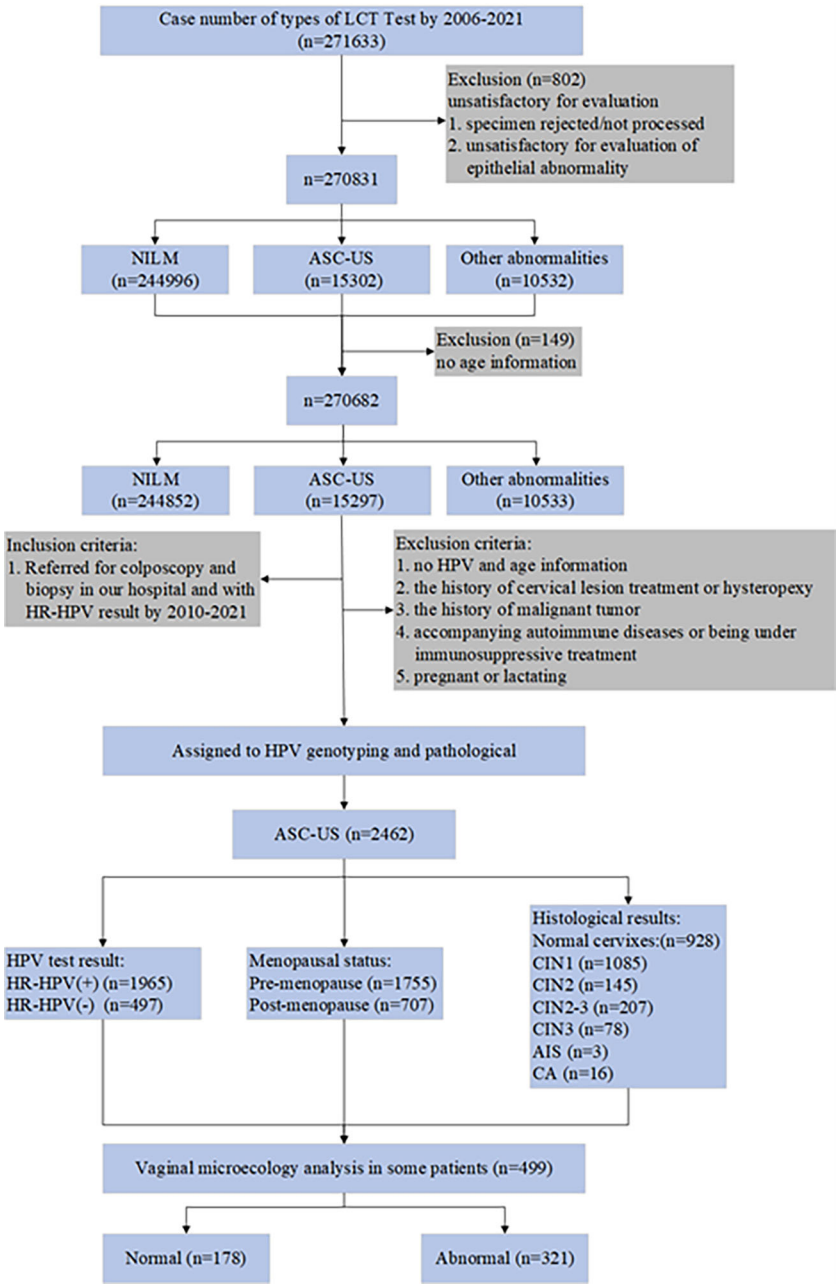

FIGURE 1
The flowchart of the study.

were diagnosed as mixed vaginitis. Examination of atrophic vaginitis revealed atrophy of the external genitalia, along with loss of the vaginal rugae. Vaginal mucosa may be friable in some areas. Microscopy of vaginal secretions showed a predominance of parabasal epithelial cells and an increased number of leukocytes. The bacteria-inhibiting flora was significantly reduced, showing no dominant bacteria with a density grade ≤ I and a diversity grade ≤ I. After excluding other types of vaginitis, a mean WBC count of ≥10/HPF in five discontinuous fields at 400 wet preparations was diagnosed as leukocytosis (Cooperative Group of Infectious Disease, Chinese Society of Obstetrics and Gynecology, Chinese Medical Association, 2016). The results of vaginal microecology detection were diagnosed by experienced physicians in the Microecology Department of Obstetrics and Gynecology of TMUGH.

2.6 Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 25.0 software for windows (SPSS Inc., Chicago, IL, USA). Chi-square test or Fisher's exact test was used for analyzing categorical variables. Statistical tests were two-sided, and $P < 0.05$ was considered statistically significant. Cochran and Mantel-Haenszel tests were performed to evaluate the

common odds ratio (OR) of the HPV-positive group compared with the HPV-negative group, controlled by pre- and post-menopause. A *P*-value < 0.05 was considered statistically significant.

3 Results

3.1 ASC-US reporting rates

During the 16-year retrospective study period, a total of 270,682 gynecologic LCT tests were performed (148 patients have been excluded due to the lack of their age-related information). ASC-US cytology was the most frequent abnormal interpretation, accounting for 5.7% of all cervical cytology in the survey (Table 1). The detection rate of ASC-US in women aged >50 years (7.0%) was significantly higher than that in women aged ≤50 years (5.0%; *P* <0.05). The ASC-US reporting rates of 7.3%, 7.1%, and 7.1% of in the age groups of 51–60 years, 61–70 years, and below 20 years, respectively, were significantly higher than the reporting rates of 5.4%, 5.1%, 4.9%, 4.5%, and 4.4% observed in the age groups of 41–50 years,71–80 years, 31–40 years, 21–30 years, and > 80 years, respectively (*P* < 0.05).

3.2 Histopathologic results and HPV test results for cases with ASC-US

A total of 2,462 eligible patients had confirmed ASC-US by cytology and were included in the study between January 2010 and December 2021, with an average age of 43.06 ± 12.27 years (range, 18–85 years). The overall HPV test results revealed that 2,381 patients had the HPV genotype, 39 had Cobas 4800, and 42 had HC2. Ultimately, there were 1,965 patients with HPV-positive status and 497 patients with HPV-negative status. The histological analysis confirmed that 928 patients (37.7%) had normal cervixes, 1,085 (44.1%) had CIN1, 145 (5.9%) had CIN2, 207 (8.4%) had CIN2–3, 78 (3.2%) had CIN3, 3(0.1%) had AIS, and 16 (0.6%) had carcinoma (including 15 squamous cell carcinomas and 1 adenocarcinoma). Among the 2,462 patients with histopathological results, diagnoses of CIN2+ were reported in 449 patients (18.2%).

The CIN2+ detection rate was significantly lower in the post- than in the pre-menopausal patients with ASC-US, as demonstrated in Table 2 (12.6 vs. 20.5%, respectively;X^2 = 21.224, *P*=0.000, <0.05).

CIN2+ lesions were found in 21.5% (422 of 1,965) of the women with ASC-US/HPV-positive results which is significantly higher than the rate of 5.4% for women (27 of 497) with ASC-US/HPV-negative results (X^2 = 68.470, *P*=0.000, <0.05). (Table 3).

In the patients with ASC-US/HR-HPV-positive status, the CIN2+ detection rate was significantly lower in the post- than in pre-menopausal patients with ASC-US, as demonstrated in Table 3 (15.0 vs. 24.0%; *P* <0.05). Furthermore, in the patients with ASC-US/HR-HPV-negative status, there was no significant difference in the detection rate of CIN2+between the post- and pre-menopausal women (X^2 = 0.514, P=0.474,*P* >0.05). The detection rates of CIN2+ in the HR-HPV-positive group were significantly higher than those in the HPV-negative group in both the pre-menopausal or post-menopausal women with ASC-US (all *P*<0.05; Table 4). In addition, Cochran and Mantel–Haenszel tests were performed to evaluate the common OR of the HR-HPV-positive group compared with the HPV-negative group in predicting the CIN2+ lesions when the menopausal status was controlled. The common OR = 4.687 (95% CI, 3.133–7.013; *P*=0.00).

A total of 475 (19.6%) patients were HPV16 positive, 147 (6.1%) were HPV18 positive, 25 (1.0%) were HPV16 and HPV18 positive, and 1,276 (52.7%) were HR HPV-positive. HPV16, HPV16 and 18 positive both showed the severity of the histological diagnosis, and the rate of CIN2+ was significantly higher than the HPV18 positive group and the HR HPV-positive group (*P* <0.05). Furthermore, the CIN2+ detection rate of the HPV16/18 positive patients with ASC-US was significantly higher than that of the other HR HPV-positive groups, *P* < 0.05. The detailed results for the pre-and post-menopausal women with ASC-US are provided in Table 5. The CIN2+ detection rates in the pre-menopausal patients with ASC-US in the HPV16 positive group, other HR HPV-positive group, and HPV16/18 positive group were significantly higher than that in the post-menopausal patients, *P* < 0.05. However, the detection rates of CIN2+ in the pre- and post-menopausal HPV18 positive group were similar (*P* >0.05).

TABLE 1 Numbers of Cervical cytology result in different age groups.

Age	No. of cervical cytology result						
	Total (No.)	NILM (No, %)		Abnormalities (No, %)		ASC-US (No, %)	
≤20	1695	1451	85.6	244	14.4	120	7.1
21-30	36035	32993	91.6	3042	8.4	1610	4.5
31-40	80710	73899	91.6	6811	8.4	3983	4.9
41-50	67378	61057	90.6	6321	9.4	3646	5.4
51-60	51823	45822	88.4	6001	11.6	3767	7.3
61-70	25173	22398	89.0	2775	11.0	1775	7.1
71-80	6835	6273	91.8	562	8.2	351	5.1
>80	1033	959	92.8	74	7.2	45	4.4
Total (No.)	270682	244852	90.5	25830	9.5	15297	5.7

TABLE 2 The histopathologic results for pre- and post-menopausal women with ASC-US.

		No. of Cases (%)		
	Average age	≤CIN1	CIN2+	Total
Pre-menopause	37.02	1395(79.50)	360(20.50)	1755
Post-menopause	58.31	618(87.4)	89(12.60)	707
P		0.00		

In the patients with ASC-US with a single infection, HPV16 was the most common HR-HPV genotype, followed, in order of decreasing frequency, by HPV52, 58, 18, and 33. The detection rate of CIN2+ in HPV16 (40.9%) was similar to that of HPV33 (29.9%; $P > 0.05$), but significantly higher than that of HPV52, 58, and 18 ($P < 0.05$). The detection rate of the CIN2 + lesions from the five most common HR-HPV genotypes (HPV16, 52, 58, 18, and 33) in women was 25.6% (227/885), which was significantly higher than that of the other single HR-HPV types (6.8%, 30/439, $P < 0.05$).

The single HPV-positive results in the pre-menopausal patients with ASC-US were as follows: HPV16, 52, 58, 18, 33, and 53. In the pre-menopausal women, the order was slightly similar.

The detection rates of CIN2+ in HPV16, 58, 18, and 33 in the pre- and post-menopausal patients with ASC-US were similar ($P > 0.05$). The detection rate of CIN2+ in HPV52 pre-menopausal patients was significantly higher than that in the post-menopausal patients ($P < 0.05$) (Table 6).

3.3 Vaginal microecology analysis results for women with ASC-US

In total, 499 patients underwent vaginal microecology analysis among the 2,462 patients with ASC-US in this study. In general, the abnormal reporting rate for vaginal microecology was 64.3% (321/499). The prevalence of abnormal reporting was significantly lower in the pre-menopausal group (56.2%) than that in the post-menopausal group (82.9%; $P<0.05$; Table 7). The rate of vaginal microecological abnormalities in the HR HPV-positive patients with ASC-US (64.3%) was similar to that in the HR HPV-negative patients with ASC-US (64.6%; $P>0.05$).

In the present study, the abnormal vaginal microecology rate of BV was the highest, accounting for 18.6% (188/448). However, the distribution of vaginal microecological abnormalities was different in the pre- and post-menopausal groups, and the prevalence of BV (19.6%), BV intermediate type (9.8%), and mixed vaginitis were relatively high in the premenopausal group, but abnormalities in the bacteria-inhibiting flora were mainly evident in the post-menopausal group, accounting for 40.8% (Table 8). There was no significant difference in the prevalence of BV, leukocytosis, intermediate type BV, trichomoniasis, and mixed vaginitis between the pre-menopausal and post-menopausal patients with ASC-US ($P > 0.05$). However, the prevalence of VVC in the pre-menopausal group was significantly higher than that in the post-menopausal group ($P<0.05$). The prevalence of AV, bacteria-inhibiting flora, and atrophic vaginitis in the post-menopausal patients was significantly higher than that the in pre-menopausal patients ($P < 0.05$).

To further explore whether the vaginal microecological abnormalities can lead to a false positive diagnosis of ASC-US, we collected the vaginal microecological results of healthy women with negative HPV and NILM in the same period, including 92 pre-menopausal and 59 post-menopausal women(Table 9). The results showed that when HPV was negative, ASC-US was evident, and histopathology revealed chronic cervicitis, the microecological abnormality rate was 66.22% (49/74), of which the post-menopausal abnormality rate was 76.92% (20/26). The microecological abnormality rate of the normal control group was 52.32% (79/151), of which the post-menopausal microecological abnormality rate was 69.50% (41/59). The abnormal rate of vaginal microecology in the normal control group was significantly lower than that in the ASC-US group ($P <0.048$), and the false-positive rate of ASC-US diagnosis increased due to the abnormal microecology. However, there was no significant difference in the rate of microecological abnormalities between the post-menopausal and pre-menopausal women.

4 Discussion

ASC-US is the most common finding among the epithelial cell abnormality, but has an equivocal cytologic diagnosis. The histopathology results of ASC-US are very different and can either be an actively proliferated benign lesion or a potentially malignant lesion (Solomon et al., 2002).

ASC-US can be characterized by (1) squamous differentiation, (2) increased nuclear-to-cytoplasmic ratio, and (3) minimal nuclear

TABLE 3 The histopathologic results for positive and negative HPV women with ASC-US.

		No. of Cases (%)		
		≤CIN1	CIN2+	Total
HPV	Positive	1543(78.5)	422(21.5)	1965
	Negative	470(94.6)	27(5.4)	497
P		0.00		

TABLE 4 Histopathologic results for pre- and post-menopausal women with ASC-US and HPV test results.

	No. of negative HPV Cases (%)			No. of positive HR- HPV Cases (%)		
	≤CIN1	CIN2+	Total	≤CIN1	CIN2+	Total
Pre-menopause	317(94.1)	20(5.9)	337	1078(76.0)	340 (24.0)[a]	1418
Post-menopause	153(95.6)	7(4.4)	160	465(85.0)	82(15.0)[a]	547
Total	470(94.6)	27(5.4)	497	1543(78.5)	422(21.5)	1965
P	0.474			0.000		

[a]P < 0.05 compared with the HPV-positive counterpart groups.

changes that may include hyperchromasia, chromatin clumping, irregularity, smudging, and/or multinucleation. Subtle and subjective findings in the specimens with ASC-US have resulted in poor reproducibility, thus compounding the difficulty in developing and illustrating strict criteria. In the case of the presence of reactive/reparative or degenerative changes, organisms, air-drying with artifactual nuclear enlargement, atrophic patterns, and atrophy of other artifacts, it is difficult to diagnose NILM or ASC-US. Thus, referral to the patient's age, medical history, and HPV results may be needed. In this study, the detection rate of ASCUS and the results of HPV, histopathology, and vaginal microecology were analyzed.

In this study, the detection rate of ASC-US was 5.7%, which is within the range of 3.7–10% of the ASC-US reporting rate in the Chinese population (Tao et al., 2015; Zheng et al., 2019; Guo et al., 2019; Tao et al., 2019; Tao et al., 2021). With age-stratified analysis, it was found that the detection rate of ASC-US in women aged 51–60, 61–70 and ≤20 years was higher than that in the women of childbearing age, which is compatible with the findings of Tao et al. (Tao et al., 2021). Women aged ≤20 years were not included in the range of cervical cancer screening according to the ASCCP guidelines. A relatively low number of women aged ≤20 years were screened, but the high detection rate of ASC-US may be due to the early age of sexual life and destruction of the cervical barrier caused by stimulation such as sexual intercourse. The average age of menopause in the Chinese women is 50 years old. In this study, the average age of menopause in the patients with ASC-US was 50.23 years old, which is comparable with the average age of menopause in the Chinese women (50 years). The estrogen levels decrease substantially with menopause, the basal layers of ectocervical epithelium become thinner, the proportion of immature basal and para basal cells increases, and the proportion of middle squamous cells decreases, which can lead to the relative enlargement of the nucleus, high nuclear–cytoplasmic ratio, hyperchromatic cells, and high nuclear–cytoplasmic ratio in the basal parathyrocytes. Many studies have confirmed that the "atrophic" form caused by low estrogen levels can increase the detection rate of ASCUS in the post-menopausal and elderly women (Cakmak and Köseoğlu, 2014).

In this study, the histopathological correlation indicated that CIN2+ and CIN3 were identified in 18.2% of the women who had ASC-US cytology. In a pooled analysis, the CIN2+ detection rate of ASC-US was 3.2% in the women aged 15–59 for initial screening, but the risk ratio greatly increased with the increase in the cumulative time and in the participants in the clinics or hospitals (Pan et al., 2014). A study conducted in the rural areas of Shanxi, China, reported that the detection rates of CIN2+ and CIN3+ in the patients with ASC-US were 7.28% and 1.75%, respectively (Wang et al., 2021). Through a systematic review, Pan et al. demonstrated that the detection rate of CIN2+ in the patients with ASC-US was 10.1% (Pan et al., 2020). Tao et al. investigated the detection rate of CIN2+ in the Chinese women with ASC-US and reported that CIN2+ was detected in 7.1% of the women with ASC-US among which 0.6% had cervical cancer (Tao et al., 2021). Similar to the observations from two other studies conducted in the United States, they found that the detection rate of cervical precancerous lesions in the patients with ASC-US was 5.1–9.7% (Stoler et al., 2011; Stoler et al., 2013). In this study, the high detection rate of CIN2+ in the patients with ASC-US may be attributed to several reasons. One reason is that some patients with ASC-US and having a HPV-negative status were not referred for colposcopic biopsy; therefore, there was an increase in the abnormal biopsy rate. Second, a small number of patients in this study had persistent ASC-US, which also increased the detection rate of CIN2+. In addition, the women with ASC-US reported in the previous studies were mostly of childbearing age or under 65 years of age. The age range in this study was large, and the rate of post-menopausal women

TABLE 5 Histopathologic results for pre- and post-menopausal women with ASC-US according to HPV genotyping.

		No. of pre-menopause Cases (%)			No. of post-menopause Cases (%)		
		≤CIN1	CIN2+	Total	≤CIN1	CIN2+	Total
negative		317(94.1)	20(5.9)	337	153(95.6)	7(4.4)	160
HPV16/18	HPV16	201(56.6)	154(43.4)	355	83(69.2)	37(30.8)	120
	HPV18	95(81.2)	22(18.8)	117	26(86.7)	4(13.3)	30
	HPV16+18	8(47.1)	9(52.9)	17	6(75.0)	2(25.0)	8
other types		747(83.7)	146(16.3)	893	345(90.1)	38(9.9)	383
Total		1368(79.6)	351(20.4)	1719	613(87.4)	88(12.6)	701

TABLE 6 Histopathologic results for pre- and post-menopausal women with ASC-US according to single HPV genotyping.

	No. of pre-menopause Cases			No. of post-menopause Cases		
	≤CIN1	CIN2+	Total	≤CIN1	CIN2+	Total
Total	748	209	957	319	48	367
HPV16	131	96[b]	227	45	23	68
HPV52	154	35[a]	189	61	4	65
HPV58	94	19 [b]	113	52	10	62
HPV18	48	16 [b]	64	19	1	20
HPV33	37	19 [b]	56	17	4	21
HPV53	45	2	47	25	0	25
HPV39	36	6	42	12	0	12
HPV56	38	2	40	20	0	20
HPV31	29	8	37	11	4	15
HPV68	36	0	36	7	0	7
HPV51	31	1	32	13	2	15
HPV59	23	2	25	18	0	18
HPV66	25	0	25	12	0	12
HPV35	10	2	12	2	0	2
HPV45	6	0	6	3	0	3
HPV73	3	0	3	0	0	0
HPV82	2	1	3	2	0	2

[a]P < 0.05 compared with the post-menopause counterpart groups.
[b]P ≥ 0.05 compared with the post-menopause counterpart groups.

with ASC-US was high, which also increased the detection rate of CIN2+. However, the relationship between ASC-US and histopathology in post-menopausal women remains controversial. Massad et al. reported that the women with ASC-US over 50 years of age had a similar detection rate of CIN2+ (about 11%) compared with those under 35 years of age, but the incidence of CIN3 and cancer was significantly higher in the older group (Massad et al., 2003). Tai et al. reported that the incidence of CIN3+ lesions in the women aged over 60 years was higher than that in the women aged between 30 and 60 years (Tai et al., 2018). Similar to other studies, the detection rate of CIN2+ in the post-menopausal women in this study was significantly lower than that in the pre-menopausal patients with ASC-US.

In the recent years, HR-HPV testing has been included in cervical cancer screening programs, which can be used to triage patients with ASC-US. According to the most current ASCCP risk-based management consensus guidelines, the immediate risk of CIN3+ is 4.4% for ASC-US/HPV-positive and 0.04% for ASC-US/HPV-negative populations (Perkins et al., 2020). Zheng et al. found that the CIN2+ lesions in 13.98% (124/887) of the women with ASC-US/HPV+ were significantly higher than those in the 2.84% (29/1022) of women with ASC-US/HPV-. They also found that cervical cancer was detected in 3.95% (35/887) of the women with ASC-US/HPV+ in the largest pathology laboratory in China (Zheng et al., 2019). The survey results of Tao et al. indicated that CIN2+ lesions were found in 657 (10.7%) of 6,154 HR-HPV-positive women with ASC-US compared with only 1.5% of HR-HPV-negative (Tao et al., 2021). In this study, CIN2+ and CIN3+ lesions were found in 21.5% and 4.6% of the women with HPV-positive/ASC-US, respectively, which were significantly higher than the women with HPV-negative/ASC-US (5.4% and 1.2%, respectively). This strongly supports the 2019 ASCCP guideline of reflex HPV testing for women with ASC-US cytology (Perkins et al., 2020). However, the risk of CIN2+ is different

TABLE 7 Vaginal microecology analysis results for women with ASC-US according to menopause state and HPV result.

	No. of Cases (%)				
		Pre-menopause	Post-menopause	HPV(+)	HPV(-)
	Total	347	152	420	79
Vaginal microecology analysis	normal	152(43.8)	26(17.1)	150(35.7)	28(35.4)
	Abnormal	195(56.2)	126(82.9)	270(64.3)	51(64.6)

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TABLE 8 Vaginal microecology analysis Results for pre- and post-menopausal Women With ASC-US.

	No. of Vaginal microecology analysis Results (%)										
	normal	BV	VVC	AV	leukocytosis	BV intermediate type	bacteria inhibiting flora	Atrophic vaginitis	Trichomoniasis	mixed vaginitis	Total
Pre-menopause	152(43.80)	68(19.60)	22(6.34)	9 (2.59)	20(5.76)	34(9.80)	10(2.88)	2 (0.58)	2 (0.58)	28(8.07)	347
Post-menopause	26(17.11)	25(16.45)	1(0.66)	10(6.58)	3(1.97)	10(6.58)	62(40.79)	9 (5.92)	0	6 (3.94)	152
Total	178(35.67)	93(18.64)	23(4.61)	19(3.81)	23(4.61)	44(8.82)	72(14.43)	11 (2.20)	2 (0.40)	34(6.81)	499
P	<0.05	>0.05	<0.05	<0.05	>0.05	>0.05	<0.05	<0.05	>0.05	>0.05	

for the women with ASC-US/HPV+ of different ages. Wang et al. showed that in the women with ASC-US/HPV+, the risk of HSIL+ in the ≤30 years old group (40.52%), 31–40 years old group (39.67%), and 41–50 years old group (34.22%) were significantly higher than that in the 51–60 years old group (21.65%), but the risk of cervical cancer was significantly higher in the women >50 years old (Wang et al., 2020a). Feng et al. reported that the risk of CIN2-3 in the perimenopausal and post-menopausal women was lower than that in pre-menopausal women with ASC-US/HPV+ (Feng et al., 2008). Similar to these investigations, in our study, the detection rate of CIN2+ in the pre-menopausal women with ASC-US/HPV+ was significantly higher than that in the post-menopausal patients, but the rates of CIN3+ and cervical cancer in the pre- and post-menopausal women were similar. Furthermore, there was no significant difference in the detection rates of CIN2+ and CIN3+ in the pre- and post-menopausal women with ASC-US/HPV-.

According to the principle of "equal management of equal risk", patients with ASC-US with different HPV genotypes should be treated differently in the clinical management. A systematic review conducted by Bonde et al. demonstrated that published evidence supports the clinical utility of HPV genotyping in risk discrimination for CIN3+ lesions during cervical cancer screening in the US, United Kingdom, Sweden, Denmark, and the Netherlands (Bonde et al., 2019). Recently, there is some experience using HR-HPV genotyping in triaging Chinese women with ASC-US.Cao et al. (2019) investigated the role of HPV 16/18 genotyping in 329 Chinese women and found that the sensitivity and specificity for HPV16/18 in detecting CIN2+ lesions in women with ASC-US were 82% and 91%, respectively (Cao et al., 2019). Wang et al. carried out a survey in the rural Shanxi province and found that compared with the 15 HR-HPV tests, genotyping for a combination of HPV16, 18, 31, 33, and 58 significantly increased the specificity with virtually no loss of sensitivity for detecting the CIN2+ and CIN3+ lesions. In addition, during the 2-year follow-up period, women with HPV16, 18, 31, 33, or 58 genotypes were the most likely population (92%, 23/25) to develop CIN2 lesion) (Wang et al., 2021). Pan et al. reported that the top five genotypes with respect to prevalence and risk of CIN2+ were HPV16, 58,18, 33, 31, and 52. The HPV16, 18, 31, 33, 52 and 58 model achieved higher sensitivity (91.3%) and specificity (70.0%) for the triage of patients with ASC-US than the other HR-HPV-type combination models (Pan et al., 2020). In the current study, we observed that the CIN2+ detection rate of HPV16/18 positive patients with ASC-US was significantly higher than that of the other high-risk HPV-positive groups, and the five highest-risk HPV genotypes were HPV16, 52, 58, 18, and 33. The five most common HPV genotypes (HPV16, 52, 58, 18, and 33) identified more CIN2+ lesions than the other HR HPV genotypes. Consistent with the results of several large studies in China, HPV16 was the most common genotype, while HPV52 and 58 were more common in the Chinese population than HPV18 (Wang et al., 2018; Zhao et al., 2018). Although HPV52 and 58 genotypes were more common than HPV 18, our results showed that the distribution of HPV genotypes in ASC-US was similar in the pre- and post-menopausal women. The most common HPV genotypes were HPV16, 52, 58, 18, and 33.

The vaginal microecology is a significant niche of the human microbiome, and the vaginal microbiota plays a significant role in the wellness maintenance of the female reproductive tract. When the

TABLE 9 Vaginal microecology analysis results for women with NILM and ASC-US.

Vaginal microecology analysis Results	NILM			ASC-US		
	Pre-menopause	Post-menopause	total	Pre-menopause	Post-menopause	total
Normal	54	18	72	19	6	25
Abnormal	38	41	79	29	20	49
Total	92	59	151	48	26	74

vagina cannot maintain its microecological balance, the acidic environment of the vagina is destroyed, and the proliferation of pathogenic bacteria replaces the initial dominance of *Lactobacillus*. The increased diversity of the bacterial community may cause upper genital tract pathological infections. Cervicitis and vaginitis have been reported as major confounding factors for the diagnosis of squamous epithelial cell abnormality (Da Silva et al., 2013). To explore the association between lower genital tract infection and cervical cytological abnormalities, Liu et al. reported that in addition to BV, Trichomoniasis, and VVC, which are the influencing factors of low-grade lesions in cells, HR-HPV infection and microecological abnormalities were mainly related to ASC-US and LSIL. In HR-HPV-negative cases, vaginal microecological abnormalities mainly caused mild morphological changes and did not lead to further cancerous changes (Liu et al., 2021). Paba et al. demonstrated that microecological abnormalities may increase the diagnosis of ASC-US and suggested that the patients with microecological abnormalities should be treated before screening for HPV and cytology (Paba et al., 2015). Our study supports that vaginal microecological abnormalities are associated with the cytological diagnosis of ASC-US.

Overall, in our study, BV was the most common microecological abnormality, which is in agreement with the previous literature (Mitra et al., 2016; Liu et al., 2021). However, the types of microecological abnormalities were different in the pre- and post-menopausal women with ASC-US, and the prevalence of the BV and BV intermediate types was relatively higher in the premenopausal women. Post-menopausal microecological abnormalities were mainly attributed to the bacteria-inhibiting flora, which accounted for 40.8% of the total cases. Furthermore, the microecological abnormality rate of post-menopausal women with ASC-US was significantly higher than that of the premenopausal women. This may be due to the reduction in the estrogen levels in the post-menopausal women, which results in decreased root colonization by *Lactobacillus* and facilitates the survival of pathogenic bacteria and viruses (Maturana et al., 2007). In addition, atrophy of the cervical epithelial cells and the high nuclear-to-cytoplasm ratio in the post-menopausal women make it difficult to distinguish them from normal cervical intraepithelial lesions. Therefore, we suggest that when cytology diagnoses ASC-US, attention should be paid to the detection of vaginal microecology. Premenopausal women should pay attention to the standard treatment for vaginitis and reduce the inflammation caused by infection to interfere with the diagnosis of ASC-US. After excluding the taboos associated with the use of estrogen, the local use of estrogen in the post-menopausal women can be suggested to reduce the shape of epithelial atrophy, and then review cytology. This can not only avoid the harm of false positives to the patient's body, mind, and economy, but also avoid a missed diagnosis.

The strength of this study is that it is the first large-scale retrospective analysis to demonstrate differences in the distribution of high-risk HPV genotypes and histopathology for ASC-US in pre- and post-menopausal women. We also demonstrated an association between the vaginal microecology and ASC-US diagnosis. However, the current study had some limitations. First, this was a retrospective study, and not all women with ASC-US were referred for colposcopic biopsy. In our study, HPV positive/ASC-US represented the majority of the patients. Accordingly, the detection rate of CIN2+ was high, which could have introduced bias in the results. In addition, not all the women in the ASC-US group had vaginal microecology, and we did not perform a stratified analysis of the symptoms of the patients of vaginitis. In future studies, the influence of HPV should be excluded and the influence of vaginal microecology on the women with ASC-US/HPV should be analyzed. Second, we did not consider the impact of HPV vaccines in this study population. However, bivalent vaccines were only approved in China in 2016; therefore, the impact of vaccines in this study population is small, but it should be a focus for further stratified analysis in the future. Therefore, large-scale prospective studies are needed to verify the distribution of HPV genotypes in women with ASC-US, differences in ASC-US among the pre- and post-menopausal women, and the impact of vaginal microecology on the diagnosis of ASC-US.

In conclusion, the detection rate of ASC-US in women over 50 years old was higher than that in women less than 50 years old, but the detection rate of CIN2+ was lower in the post-menopausal women than in the premenopausal women with ASC-US. However, the vaginal microecological abnormalities may increase the false-positive diagnosis rate of ASC-US. The vaginal microecological abnormalities of menopausal women with ASC-US are mainly infectious diseases such as BV which occurs in post-menopausal women and are mainly caused by the decrease in the bacteria-inhibiting flora due to mucosal epithelial atrophy. Therefore, more attention should be paid to the detection of vaginal microecology to avoid the high referral rate for colposcopy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Committee Tianjin Medical University General

Hospital Tianjin, China. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

AF, and FX conceived the study question, and all authors were involved in the study design. YY was involved in the statistical analysis while BL, LD, and CW interpreted the results. BL created the first draft of the manuscript. BL, LD, CW, JL, XZ, AF, and FX made substantial contributions to drafting the article and revising it critically. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abdulaziz, A., Liu, L., Sun, Y., You, X., Cui, B., Han, S., et al. (2020). Clinicopathologic significance and treatment of ASC-US in cervical cytology. *Int. J. Clin. Exp. Pathol.* 13 (2), 307–316.

Bhatla, N., Singhal, S., Saraiya, U., Srivastava, S., Bhaleral, S., Shamsunder, S., et al. (2020). Screening and management of preinvasive lesions of the cervix: Good clinical practice recommendations from the federation of obstetrics and gynaecologic societies of India (FOGSI). *J. Obstet. Gynaecol. Res.* 46 (2), 201–214. doi: 10.1111/jog.14168

Bonde, J. H., Sandri, M. T., Gary, D. S., and Andrews, J. C. (2019). "Clinical utility of human papillomavirus genotyping in cervical cancer screening: A systematic review". *J. Low. Genit. Tract Dis.* 24 (1), 1–13. doi: 10.1097/LGT.0000000000000494

Brotman, R. M., Shardell, M. D., Gajer, P., Tracy, J. K., Zenilman, J. M., Ravel, J., et al. (2014). Interplay between the temporal dynamics of the vaginal microbiota and human papillomavirus detection. *J. Infect. Dis.* 210 (11), 1723–1733. doi: 10.1093/infdis/jiu330

Bruno, M. T., Coco, A., Di Pasqua, S., and Bonanno, G. (2019). Management of ASC-US/HPV positive post–menopausal woman. *Virol. J.* 16 (1), 39. doi: 10.1186/s12985-019-1145-5

Cakmak, B., and Köseoğlu, D. R. (2014). Comparison of cervical cytological screening results between post-menopausal and elderly women. *Turk Patoloji Derg.* 30 (1), 38–42. doi: 10.5146/tjpath.2013.01212

Cao, X., Liu, S., Jia, M., Chen, H., Zhan, D., Domg, B., et al. (2019). Performance of HPV16/18 in triage of cytological atypical squamous cells of undetermined significance. *Anal. Cell. Pathol. (Amst).* 2019, 4324710. doi: 10.1155/2019/4324710

Cooperative Group of Infectious Disease, Chinese Society of Obstetrics and Gynocology, Chinese Medical Association. (2016). Expert consensus on the clinical application of vaginal microecology test. *Zhonghua Fu Chan Ke Za Zhi.* 51 (10), 721–723. doi: 10.3760/cma.j.issn.0529-567X.2016.10.001

Da Silva, M. G., De Almeida, R. T., Bastos, E. A., and Nobre, F. F. (2013). Determinants of cellular atypia detection in the cervical screening program in Rio de Janeiro, Brazil. *Rev. Panam Salud Publica.* 34 (2), 107–113.

Donders, G. G. G., Bellen, G., Grinceviciene, S., Ruban, K., and Vieira-Baptista, P. (2017). Erobic vaginitis: No longer a stranger. *Res. Microbiol.* 168 (9-10), 845–858. doi: 10.1016/j.resmic.2017.04.004

Feng, J., Al-Abbadi, M. A., Bandyopadhyay, S., Salimnia, H., and Husain, M. (2008). Significance of high-risk human papillomavirus DNA-positive atypical squamous cells of undetermined significance pap smears in perimenopausal and post-menopausal women. *Acta Cytol.* 52 (4), 434–438. doi: 10.1159/000325548

Gomes de Oliveira, G., Eleutério, R. M. N., Silveira Gonçalves, A. K., Giraldo, P. C., and Eleutério, J. (2018). Atypical squamous cells in liquid-based cervical cytology: Microbiology, inflammatory infiltrate, and human papillomavirus-DNA testing. *Acta cytol.* 62 (1), 28–33. doi: 10.1159/000481654

Guo, Z., Jia, M., Chen, Q., Chen, H., Chen, P., Zhao, D., et al. (2019). Performance of different combination models of high-risk HPV genotyping in triaging Chinese women with atypical squamous cells of undetermined significance. *Front. Oncol.* 9. doi: 10.3389/fonc.2019.00202

Holt, H. K., Zhang, L., Zhao, F. H., Hu, S. Y., Zhao, X. L., Zhang, X., et al. (2017). Evaluation of multiple primary and combination screening strategies in post-menopausal women for detection of cervical cancer in China. *Int. J. Cancer* 140 (3), 544–554. doi: 10.1002/ijc.30468

Koh, W. J., Abu-Rustum, N. R., Bean, S., Bradley, K., Campos, S. M., Cho, K. R., et al. (2019). Cervical cancer, version 3.2019, NCCN clinical practice guidelines in oncology. *J. Natl. Compr. Canc. Netw.* 17 (1), 64–84. doi: 10.6004/jnccn.2019.0001

Kyrgiou, M., Arbyn, M., Bergeron, C., Bosch, F. X., Dillner, J., Jit, M., et al. (2020). Cervical screening: ESGO-EFC position paper of the European society of gynaecologic oncology (ESGO) and the European federation of colposcopy (EFC). *Br. J. Cancer.* 123 (4), 510–517. doi: 10.1038/s41416-020-0920-9

Liu, Y., Li, T., Wang, S., Chen, T., Wu, D., Liu, B., et al. (2021). Association between lower genital tract infection and cervical cytological abnormalities. *Chin. J. Clin. Obstet. Gynecol.* 22 (6), 601–605. doi: 10.13390/j.issn.1672-1861.2021.06.012

Massad, L. S., Behbakht, K., Collins, Y. C., and Cejtin, H. E. (2003). Histologic findings from the cervix among older women with abnormal cervical cytology. *Gynecol. Oncol.* 88 (3), 340–344. doi: 10.1016/s0090-8258(02)00135-x

Maturana, M. A., Irigoyen, M. C., and Spritzer, P. M. (2007). Menopause, estrogens, and endothelial dysfunction: Current concepts. *Clinics (Sao Paulo)* 62 (1), 77–86. doi: 10.1590/s1807-59322007000100012

Mitra, A., MacIntyre, D. A., Marchesi, J. R., Lee, Y. S., Bennett, P. R., and Kyrgiou, M. (2016). The vaginal microbiota, human papillomavirus infection and cervical intraepithelial neoplasia: what do we know and where are we going next? *Microbiome* 4 (1), 58. doi: 10.1186/s40168-016-0203-0

Nayar, R., and Wilbur, D. C. (2015). *The Bethesda system for reporting cervical cytology* (New York Dordrecht London: Springer International Publishing Switzerland).

Paba, P., Criscuolo, A. A., Grazia Giancipoli, R., Santi, F., Ascone, C., Sesti, F., et al. (2015). Microbiological infections in women with cervical cytological reports of atypical

squamous cells of undetermined significance. *J. Low. Genit. Tract Dis. Vol.* 19 (3), 203–206. doi: 10.1097/LGT.0000000000000078

Pan, D., Dong, B., Gao, H., Mao, X., Xue, H., and Sun, P. (2020). The triage effectiveness of an extended high-risk human papillomavirus genotyping assay for women with cytology showing atypical squamous cells of undetermined significance in China. *Risk Manage. Healthc. Policy.* 13, 1747–1756. doi: 10.2147/RMHP.S270265

Pan, Q. J., Hu, S. Y., Guo, H. Q., Zhang, W. H., Zhang, X., Chen, W., et al. (2014). Liquid-based cytology and human papillomavirus testing: A pooled analysis using the data from 13 population-based cervical cancer screening studies from China. *Gynecol. Oncol.* 133 (2), 172–179. doi: 10.1016/j.ygyno.2014.03.008

Peres, A. L., Camarotti, J. R., Cartaxo, M., Alencar, N., Stocco, R. C., Beçak, W., et al. (2015). Molecular analysis and conventional cytology: Association between HPV and bacterial vaginosis in the cervical abnormalities of a Brazilian population. *Genet. Mol. Res.* 14 (3), 9497–9505. doi: 10.4238/2015.August.14.13

Perkins, R. B., Guido, R. S., Castle, P. E., Chelmow, D., Einstein, M. H., Garcia, F., et al. (2020). 2019 ASCCP risk-based management consensus guidelines for abnormal cervical cancer screening tests and cancer precursors. *J. Low. Genit. Tract Dis.* 24 (2), 102–131. doi: 10.1097/LGT.0000000000000525

Plisko, O., Zodzika, J., Jermakova, I., Pcolkina, K., Prusakevica, A., Liepniece-Karele, I., et al. (2021). Aerobic vaginitis–underestimated risk factor for cervical intraepithelial neoplasia. *Diagnostics (Basel)* 11 (1), 97. doi: 10.3390/diagnostics11010097

Solomon, D., Davey, D., Kurman, R., Moriarty, A., O'Connor, D., Prey, M., et al. (2002). The 2001 bethesda system: Terminology for reporting results of cervical cytology. *JAMA* 287 (16), 2114–2119. doi: 10.1001/jama.287.16.2114

Stoler, M. H., Wright, T.Jr, Cuzick, J., Dockter, J., Reid, J. L., Getman, D., et al. (2013). APTIMA HPV assay performance in women with atypical squamous cells of undetermined significance cytology results. *Am. J. Obstet. Gynecol.* 208 (2), 144.e1–144.e1448. doi: 10.1016/j.ajog.2012.12.003

Stoler, M. H., Wright, T.Jr, Sharma, A., Apple, R., Gutekunst, K., Wright, T. L., et al. (2011). High-risk human papillomavirus testing in women with ASC-US cytology: Results from the ATHENA HPV study. *Am. J. Clin. Pathol.* 135 (3), 468–475. doi: 10.1309/AJCPZ5JY6FCVNMOTs

Tai, Y. J., Chen, Y. Y., Hsu, H. C., Chiang, C. J., You, S. L., Chen, C. A., et al. (2018). Risks of cervical intraepithelial neoplasia grade 3 or invasive cancers in ASCUS women with different management: A population-based cohort study. *J. Gynecol. Oncol.* 29 (4), e55. doi: 10.3802/jgo.2018.29.e55

Tao, X., Austin, R. M., Kong, L., Sun, Q., Lv, Q., Xu, H., et al. (2019). Nationwide survey of cervical cytology laboratory practices in China. *J. Am. Soc Cytopathol.* 8 (5), 250–257. doi: 10.1016/j.jasc.2019.02.002

Tao, X., Austin, R. M., Zhang, H., Zhang, L., Xiao, J., Wang, L., et al (2015). Pap test reporting rates for conventional smear and liquid-based cervical cytology from the largest academic women's hospital in China: Analysis of 1,248,785 pap test reports". *Acta cytol.* 59 (6), 445–451. doi: 10.1159/000443679

Tao, X., Zhang, H., Wang, L., Pan, Q., Ji, S., Zhou, X., et al. (2021). Atypical squamous cells of undetermined significance cervical cytology in the Chinese population: Age-stratified reporting rates, high-risk HPV testing, and immediate histologic correlation results. *Cancer Cytopathol.* 129 (1), 24–32. doi: 10.1002/cncy.22333

Wang, Z., Gu, Y., Wang, H., Chen, J., Zheng, Y., Cui, B., et al. (2020). Distribution of cervical lesions in high-risk HPV (hr-HPV) positive women with ASC-US: A retrospective single-center study in China. *Virol. J.* 17 (1), 185. doi: 10.1186/s12985-020-01455-2

Wang, Z., Li, Z., Li, J., Wang, C., Wang, W., Hao, M., et al. (2018). Prevalence and distribution of HPV genotypes in 1387 women with cervical intraepithelial neoplasia 2/3 in shanxi province, China. *J. Cancer.* 9 (16), 2802–2806. doi: 10.7150/jca.25614

Wang, Z., Wang, T., Yang, J., Wang, W., Zhang, L., Su, X., et al. (2020). Diagnostic yield and performance of a large population-based cervical cancer screening program in high-risk rural China. *J. Cancer.* 11 (13), 4000–4006. doi: 10.7150/jca.41472

Wang, W., Zhang, H., Lin, L., Yang, A., Yang, J., Zhao, W., et al. (2021). Efficient combination of human papillomavirus genotyping for the triage of women with atypical squamous cells of undetermined significance in Chinese rural population: A population-based study. *J. Cancer.* 12 (10), 2815–2824. doi: 10.7150/jca.55771

Watson, M., Benard, V., Lin, L., Rockwell, T., and Royalty, J. (2015). Provider management of equivocal cervical cancer screening results among underserved women 2009-2011: follow-up of atypical squamous cells of undetermined significance. *Cancer Causes Control.* 26 (5), 759–764. doi: 10.1007/s10552-015-0549-9

Workowski, K. A., Bachmann, L. H., Chan, P. A., Johnston, C. M., Muzny, C. A., Park, I., et al. (2021). Sexually transmitted infections treatment guidelines 2021. *MMWR Recomm. Rep.* 70 (4), 1–187. doi: 10.15585/mmwr.rr7004a1

Xu, S., Lan, Y., Ma, L., and Zhou, J. (2021). Changes of vaginal microecology and related research progress in post-menopausal women. *Chin. J. Geriatr.* 12), 401–405. doi: 10.3760/cma.j.issn.0254-9026.2021.03.028. 2021.

Zeferino, L. C., Bastos, J. B., Vale, D. B. A. P. D., Zanine, R. M., Melo, Y. L. M. F., Primo, W. Q. S. P., et al. (2018). Guidelines for HPV-DNA testing for cervical cancer screening in brazil. *Rev. Bras. Ginecol. Obstet.* 40 (6), 360–368. doi: 10.1055/s-0038-1657754

Zhao, Y. Q., Dai, Y., Dang, L., Kong, L. H., Zhang, Y., Feng, R. M., et al. (2018). Real-world research on cervical cancer screening program and effect evaluation for Chinese population. *Zhonghua zhong liu za zhi [Chin. J. Oncol.]* 40 (10), 764–771. doi: 10.3760/cma.j.issn.0253-3766.2018.10.008

Zheng, B., Yang, H., Li, Z., You, J., Wei, G., Zhang, H., et al. (2019). Atypical squamous cells of undetermined significance cervical cytology report rate and histologic follow-up findings from the largest college of American pathologists-certified laboratory in China. *Arch. Pathol. Lab. Med.* 143 (6), 748–752. doi: 10.5858/arpa.2018-0244-OA

Zheng, R., Zhang, S., Zeng, H., Wang, S., Sun, K., Chen, R., et al. (2022). Cancer incidence and mortality in China 2016. *J. Natl. Cancer Cent.* 2 (1), 1–9. doi: 10.1016/j.jncc.2022.02.002

Zhou, H. L., Zhang, W., Zhang, C. J., Wang, S. M., Duan, Y. C., Wang, J. X., et al. (2018). Prevalence and distribution of human papillomavirus genotypes in Chinese women between 1991 and 2016: A systematic review". *J. Infect.* 76 (6), 522–528. doi: 10.1016/j.jinf.2018.02.008

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Bingbing Xiao,
First Hospital, Peking University, China
Xinyue Hu,
Nanchang University, China
Long Sui,
Fudan University, China

*CORRESPONDENCE
Cha Han
tjhancha@163.com
Fengxia Xue
xuefengxia@tmu.edu.cn

†These authors have contributed equally to this work

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Interactions between microbiota and cervical epithelial, immune, and mucus barrier

Mengting Dong[1,2†], Yalan Dong[1,2†], Junyi Bai[1,2], Huanrong Li[1,2], Xiaotong Ma[1,2], Bijun Li[1,2], Chen Wang[1,2], Huiyang Li[1,2], Wenhui Qi[1,2], Yingmei Wang[1,2], Aiping Fan[1,2], Cha Han[1,2*] and Fengxia Xue[1,2*]

[1]Department of Obstetrics and Gynaecology, Tianjin Medical University General Hospital, Tianjin, China, [2]Tianjin Key Laboratory of Female Reproductive Health and Eugenic, Department of Gynecology and Obstetrics, Tianjin Medical University General Hospital, Tianjin, China

The female reproductive tract harbours hundreds of bacterial species and produces numerous metabolites. The uterine cervix is located between the upper and lower parts of the female genital tract. It allows sperm and birth passage and hinders the upward movement of microorganisms into a relatively sterile uterus. It is also the predicted site for sexually transmitted infection (STI), such as Chlamydia, human papilloma virus (HPV), and human immunodeficiency virus (HIV). The healthy cervicovaginal microbiota maintains cervical epithelial barrier integrity and modulates the mucosal immune system. Perturbations of the microbiota composition accompany changes in microbial metabolites that induce local inflammation, damage the cervical epithelial and immune barrier, and increase susceptibility to STI infection and relative disease progression. This review examined the intimate interactions between the cervicovaginal microbiota, relative metabolites, and the cervical epithelial-, immune-, and mucus barrier, and the potent effect of the host-microbiota interaction on specific STI infection. An improved understanding of cervicovaginal microbiota regulation on cervical microenvironment homeostasis might promote advances in diagnostic and therapeutic approaches for various STI diseases.

KEYWORDS

cervicovaginal microbiota, uterine cervix, epithelial, immune, mucus, chlamydia trachomatis, human papilloma virus, human immunodeficiency virus

Introduction

Cervicovaginal microbiota have a mutual relationship with the host. The lower genital tract of women provides an ecological niche for bacterial colonisation. *Lactobacillus* spp. predominates the microbial flora in a healthy female genital tract, which confers several benefits to the host. It ferments the glucose and maltose of the vaginal epithelial cells to produce lactic acid which maintains vaginal pH at 3.8~4.5. Lactic acid and its associated

acidic microenvironment help regulate inflammation. *Lactobacilli* also secrete bacteriocin (Scillato et al., 2021), which inhibits the proliferation of other microorganisms and has strong epithelial cell adhesion ability, thereby competing with pathogens for living space and nutrient uptake (Kwok et al., 2006). Female genital microbiota can be tightly regulated by host factors such as age, menstrual cycle, sex steroid hormones, race, living habits, immunity, and genetic polymorphism. Microbial metabolites, components, and bacteria can interact with host epithelial cells and resident immune cells, leading to alterations in the local ecosystem and affecting defences against pathogen infection and disease progression. The dominant *Lactobacilli* flora maintains the health of the reproductive tract, whereas dysbiosis of the microbiota, wherein Lactobacilli are significantly reduced or absent and replaced by pathogenic bacteria is indicative of the development of reproductive tract-associated diseases (Smith and Ravel, 2017).

The female reproductive tract (FRT) can be divided into the lower FRT (vagina and ectocervix) which contains a large microbial presence, and the upper FRT (endocervix, uterus, and oviduct) which is relatively sterile. The cervix includes the ecto- and endocervix and plays a specific role in FRT. On the one hand, the cervix acts as a channel through which human sperm can migrate into the uterus, and as an infant passage after dramatic changes during gestation and parturition. On the other hand, the cervix and mucus should prevent microorganisms in the lower reproductive tract which may induce pelvic inflammatory disease (PID). It is also the site of attack of specific sexually transmitted infection (STI) pathogens including *Chlamydia trachomatis* (CT), human papillomavirus (HPV), and human immunodeficiency virus (HIV).

A healthy cervicovaginal microbiota (CVM) and the acidic environment formed by the associated metabolites maintain cervical epithelial barrier integrity, stabilise the mucosal immune system, and balance host defence and tolerance with microbes, metabolites, components, and attachment to host cells. In contrast, microbiota dysbiosis and its accompanying changes in microbial metabolites induce an inflammatory response, cause damage to cervical cells, disrupt cervical epithelial and mucosal barriers, stimulate the immune system, and cause an imbalance in cervical homeostasis, thereby inducing various STI diseases. This review highlights recent advances in the understanding of the interactions between the microbiota and the cervical mucosal barrier (epithelial, mucus, and immune), and discusses the implications for cervical-specific STIs.

Normal physiology state

Anatomy and histology of the cervix

The cervix is a cylindrical structure approximately 2-cm long, with the cervical canal in the centre, the inner orifice opening to the uterus, and the outer opening to the vagina. The cervix is divided into the ectocervix and endocervix depending on whether it is exposed to the vagina. The cervix is composed of stroma and epithelial cells separated by a basement membrane. The underlying stroma of the cervix is predominately an extracellular matrix, including type I and type II collagen, and a small amount of type IV collagen (Leppert, 1995). Approximately 70% of the cervix's extracellular matrix is composed of type I collagen, which plays a crucial role in maintaining tissue integrity (Yellon, 2019). Type IV collagen can be found in the basement membrane as well as enriched in the placenta (Lithgow et al., 2022). Other components included elastin, proteoglycans, hyaluronan, fibroblasts, and scattered immune cells (Tantengco et al., 2021a). Epithelial cells are region-specific, and the endocervix is covered by columnar epithelial cells. The ectocervix and vagina are covered by continuous stratified non-keratinised squamous epithelial cells. The area between the endocervix and ectocervix is the transformation zone, which is covered by squamous columnar epithelial cells. Intercellular junctions and the epithelial cells formed cervical epithelial barrier. The main connections between epithelial cells include tight junctions, adherent junctions, and desmosome junctions (Blaskewicz et al., 2011). Columnar epithelial cells are mainly composed of tight junctions that are directly regulated by oestrogens, cytokines, and growth factors (Grant and Wira, 2003; Wira et al., 2010). The squamous epithelium mainly consists of adhesive junctions and desmosome junctions, while tight junctions are lacking, allowing small molecules to pass through the intercellular space. Pathogenic microorganisms can contact Langerhans cells and $CD4^+$ T cells distributed in the cervix (Hladik et al., 2007). In addition, epithelial cells can act as antigen-presenting cells (APCs) to recognise pathogenic microorganisms and secrete immune components such as antibiotics, cytokines, and chemokines.

Cervical mucus barrier

Cervical mucus is produced by goblet/secretory cells around the crypts in the endocervical canal (Han et al., 2017) and contains mucins (MUCs), immunoglobulins, plasma proteins, lipids, sterols, carbohydrates, inorganic ions, and water. Mucin is a large O-linked glycosylated protein and is the main component of the cervical mucus (Radtke et al., 2012). Currently, 20 mucins are known, which are divided into gels such as MUC5AC, MUC5B, and MUC6, transmembrane proteins such as MUC1 and MUC16, and small soluble molecules (Andersch-Björkman et al., 2007; Radtke et al., 2012). MUC5B and MUC5AC are the main mucins of the cervix (Gipson et al., 1999; Gipson et al., 2001), and MUC5B is the main mucin that affects the properties of the cervical mucus. During the follicular phase, the amount of mucus increases with increasing oestrogen levels, and the mucus gradually becomes thinner. During ovulation, the mucus levels increase by 10–20 times, MUC5B expression reaches its highest level, and the secretion becomes watery, which facilitates the entry of sperm into the upper reproductive tract (Han et al., 2021). During the luteal phase, MUC5B expression decreases owing to the effect of progesterone, and the cervical mucus gradually thickens (Gipson et al., 2001). The levels of immune factors such as IgG/IgA, lactoferrin, interleukin-10 (IL-10), and antimicrobial peptides also show a bimodal distribution with the menstrual cycle (Ulcova-Gallova, 2010), with higher levels in the early stage of the follicular phase, followed by 10-1,000 times decreases in the middle of menstruation, and increases in the later stage of menstruation. However, the levels of IFN-γ, IL-4, IL-6, and IL-12 did not show periodic changes (White et al., 1997; Han et al.,

2017). Hughes performed a meta-analysis include 31 article mainly CVL samples, found that antibodies, CC–type chemokines, MMPs, IL–6, IL–16, IL–1RA, G–CSF, GNLY, and ICAM1 were lower in the luteal phase than the follicular phase. IL–1α, HBD–2, and HBD–3 were elevated in the luteal phase. CXCL8, 9, and 10, interferons, TNF, SLPI, elafn, lysozyme, lactoferrin, and IL-1β, 2, 10,12, 13, and 17A show minimal change. Although some constituents showing cyclic variations increase the vulnerability of sperm passaging and pathogen ascent, some cytokines did not change with the cell cycle which may compensate for the risk of ascending infection (mucus increase) during ovaluation (De Tomasi et al., 2019).

The cervical mucus can play a barrier role in both physical and immunological aspects. First, mucin and other substances capture pathogenic microorganism particles and slow down the diffusion speed to gain time for local immune responses (Boukari et al., 2009; Shukair et al., 2013). Second, immune factors such as IgG, IgA, lactoferrin, and SLPI in cervical mucus can inhibit the adhesion and invasion of cervical epithelial cells by pathogenic microorganisms such as CT, and HIV (Radtke et al., 2012; Filardo et al., 2019).

Distribution of local immune cells in the cervix

Studies on cervical immune cells mainly focus on cervical tissue and secretion samples. Neutrophils are the most prevalence immune cells found in cytobrush secretion samples, supported by studies from non-pregnant- (Mohammadi et al., 2020) and pregnant women (Hunter et al., 2016); this differs from samples collected from biopsies (Trifonova et al., 2014). Both cervical and vaginal tissues are mainly composed of APCs and T cells, and most of them are effector memory- or effector T cells, indicating that the cervical region is an organ dominated by cellular immunity (Trifonova et al., 2014). The content of APCs and T cells is higher in the cervix than those in the vagina, with the transformation zone and endocervix having the largest and smallest amounts, respectively (Pudney et al., 2005). In the ectocervix, 37–55% are APC cells (most are macrophages), followed by dendritic cells (DCs), and 23–43% are T lymphocytes. The number of APCs in the endocervix is similar to that in the ectocervix, however, T lymphocytes are approximately half of those in the ectocervix (Trifonova et al., 2014). $CD8^+$ T cells are slightly more abundant than $CD4^+$ T cells, with the latter accounting for approximately 50% of the total $CD3^+$ cells in the endocervix and ectocervix. Th17 cells with CCR5 and CD90 co-expression are the main $CD4^+$T cell population in the cervix (Rodriguez-Garcia et al., 2014).

Human cellular immunity works through the following processes: local APCs recognise pathogenic microorganisms, present antigens to lymphoid tissues, and T cells are stimulated and differentiate into effector T cells in local tissues. Subsequently, some effector T cells develop into memory T cells, which are divided into central memory T cells (TCM) that are stored in the extralymphatic tissue, and effector memory T cells (TEM) which can travel between the blood and extralymphatic tissue and play a role in circulating immune surveillance. Recent work found that some memory T cells also reside in local tissues and become tissue-resident memory T cells (TRMs) (Gebhardt et al., 2013; Sathaliyawala et al., 2013). An increasing number of studies confirmed that circulating memory cells fail to approach the genital tract mucosa at a steady state, while TRMs can quickly play a local immune role without being recirculated through lymphoid tissue (Iijima and Iwasaki, 2014). $CD8^+$TRM ($CD69^+CD103^+$) is the predominant fraction in $CD8^+$T cells of cervicovaginal tissue, while others are $CD69^+$ single positive and double negative $CD69^-CD103^-$ cells, which are also defined as inflammatory mucosal T cells (Tim) (Pattacini et al., 2019). Most $CD8^+$TRMs in the mouse reproductive tract express CD103, which binds to E-cadherin and promotes TRM retention within certain epithelial compartments (Rosato et al., 2017). Only a few $CD4^+$TRMs express CD103, and $CD4^+$TRMs are mainly distributed within memory lymphocyte clusters (MLCs). Once TRM recognises the cognate peptide, it releases cytokines IL-2, TNFα, and IFNγ, thereby upregulating adhesion molecules and chemokines and promoting the recruitment of circulating memory T cells and B cells into tissues (Schenkel et al., 2014; Rosato et al., 2017).

Studies on the effects of the menstrual cycle and menopause on cervical lymphocytes are scarce and controversial. Several studies found that the total number of $CD8^+$T and $CD4^+$T cells in the cervix does not change with the menstrual cycle and menopause (Pudney et al., 2005), and the toxicity of $CD8^+$T cells is unaffected (Rodriguez-Garcia et al., 2020). In addition, endometrial $CD8^+$T cells are inhibited by an increase in transforming growth factor beta (TGF-β) in the secretory phase, but cervical $CD8^+$T cells are insensitive to TGF-β regulation. This means that the cervix can still play an immunoprotective role in preventing pathogenic microorganisms from ascending when the secretory endometrium is in an immunosuppressed state due to embryo implantation (Rodriguez-Garcia et al., 2020). However, other studies show that the menstrual cycle and age can affect the number and function of certain lymphocyte subsets. For example, the level of chemokine (C-C motif) ligand 2 CCL2 and the proportion of local $CD4^+$TRM in the cervix increases during the follicular period (Boily-Larouche et al., 2019). CCL2 is a chemokine found in monocytes/macrophages, T cells, and TRM. The inhibitory effect of progesterone on CCL2 decreases during the follicular phase, and more $CD4^+$TRMs are recruited to the local cervix (Hall and Klein, 2017; Boily-Larouche et al., 2019). In addition, the abundance of $CD8^+$TRM and DC cells in the cervical tissue of postmenopausal women decreases with age (Rodriguez-Garcia et al., 2018). This may be the reason for the increased susceptibility of postmenopausal women to STI (Ghosh et al., 2014) and the second peak of HPV infection at the age of 45 (Rodriguez-Garcia et al., 2018).

Normal cervicovaginal microbiome

The microbiota of the lower genital tract is identified more frequently than that of the endocervix. Using self-collected vaginal secretions samples, five “communities state types” (CSTs) are classified according to the composition and proportion of vaginal microbiota by Ravel et al. Specifically, CST-I, CST-II, CST-III, and CST-V corresponds to a predominance of *Lactobacillus crispatus, L. gasseri, L. iners*, and *L. jensenii*, respectively, whereas CST-IV is characterised by a combination of various facultative anaerobes with a low abundance of *Lactobacilli*. CST IV-A comprises *Anaerococcus, Prevotella, Corynebacterium, Peptoniphilus,*

Finegoldia, and *Streptococcus*. CST IV-B is characterised by *Atopobium, Gardnerella, Mobiluncus, Sneathia, Megasphaera*, and other bcteria in the order *Clostridiales* (Ravel et al., 2011). In 2020, they comprehensively re-identified common vaginal CSTs in 1975 women with different ethnicities; black, white, Hispanic, and Asian. CST IV is divided into CST IV-A, IV-B, and IV-C. CST IV-A has a high level of *Candidatus Lachnocurva vaginae* (formerly called bacterial vaginosis-associated bacterium-1, BVAB1) and a moderate amount of *Gardnerella vaginalis*, whereas IV-B has a high abundance of *G. vaginalis* and a low amount of *Ca. L. vaginae*. Both IV-A and IV-B contain moderate levels of *Atopobium vaginae*. CST IV-C is characterised by other diverse anaerobic bacteria and is divided into five sub-CSTs: CST IV-C0 is a homogeneous community with a moderate level of *Prevotella* spp., CST IV-C1, C2, C4 with *Streptococcus* spp., *Enterococcus* spp., *Staphylococcus spp*, as a dominated flora respectively. CST IV-C3 with *Bifidobacterium* spp. as dominant flora (France et al., 2020). In addition, "cervicotype" (CT) was proposed by Anathar et al. (Anahtar et al., 2015) by analysing the microbiota community structure of ectocervical swabs. CT1 is primarily dominated by non-iners *Lactobacillus*, CT2 is dominated by *L. iners*, CT3 by *Gardnerella*, and CT4 by diverse anaerobes associated with BV. Buck et al. further defined the structure of vaginal flora with "vagitype" (Fettweis et al., 2019). Although both vagitypes and dominant bacteria refer to bacterial species with a relative abundance of over 30%, each sample can have over two dominant bacterial species, and vagitypes refer to the most dominant and functional bacterial species. Normal vaginal flora is often composed of only one or two dominant bacteria due to the strong adhesion ability of *Lactobacillus*.

The microbiota in the vagina, endocervix, and uterine cavity of the same female show continuity. The overall microbiota biomass shows a downward trend and bacterial diversity gradually increases. The microbiota in the endocervix is more similar to the vagina than the uterus and is dominated by *Lactobacillus*. However, the proportion of *Lactobacillus* in the endocervix is lower than that in the vagina, and the relative abundance of *Bacteroides, Pseudomonas*, and *Prevotella* spp. increases. The relative abundance of *Lactobacillus* in the uterus is lower than in the vagina and endocervix (Chen et al., 2017; Zhang et al., 2021; Wang et al., 2021a). It is suggested that microflora quantity and composition is altered with the rise in genital tract position. The cervix is located between the vagina and uterus, and the characteristics of its microflora are between those of the vagina and uterus, suggesting that it is the transformation zone of bacteria in the reproductive tract.

CVM can be dynamic with the menstrual cycle and age. During menses, increased diversity (Krog et al., 2022) and decreased *Lactobacillus* abundances (Song et al., 2020) were observed. During the follicular and luteal phases, the abundance of *Lactobacillus* is expanded, and the vaginal microbiome of more women becomes dominated by *Lactobacillus* due to the increase of estradiol (Gajer et al., 2012; Song et al., 2020; Jie et al., 2021; Krog et al., 2022; Oerlemans et al., 2022). In postmenopausal women, species richness was decreased, but species diversity was significantly increased (Kim et al., 2021). The abundance of lactobacillus is decreased, while the proportion of anaerobic bacteria is increased (Gliniewicz et al., 2019; Kim et al., 2021). The composition of these communities resembles those of premenopausal women with BV and is correlated with symptoms of vaginal discomfort (Shardell et al., 2021; Mitchell et al., 2021a). However, the bacteria associated with BV nugent score in postmenopausal women were different from premenopausal women, which demonstrated there are differences in microbial community with premenopausal women with BV (Mitchell et al., 2021b).

Many studies also demonstrate that CVM is associated with disease treatment outcomes. For example, some studies reveal that low baseline *Lactobacillus* (Xiao et al., 2022), *L.iners*.(Zhou et al., 2022), high *G.vaginalis* and *A. vaginae* (Bradshaw et al., 2006; Ferreira et al., 2017) was associated with BV treatment failure and recurrence, although some reports show inconsistent effect. The discrepancy is partly due to different study designs, populations and technology. In addition, the different virulence of species (Mollin et al., 2022; Zhou et al., 2022), and the dynamics of the species in CVM (Xiao et al., 2019), rather than the genus as a whole maybe provide more information to monitor the treatment outcome.

Interaction between microbiota and cervical barrier

Microbiome dysbiosis and metabolite alterations

The microbiota and metabolites changed during cervicovaginal dysbiosis. *Lactobacilli* are the dominant species in healthy conditions and exclusively utilise sugars such as glycogen and glycogen hydrolysates as carbon and nitrogen sources to produce lactic acid (Amabebe and Anumba, 2018). *Lactobacilli* produce lactic acid, bacteriocins, and biosurfactants to protect the cervicovaginal environment. Bacterial vaginosis (BV) is one of the most common dysbiosis in the vagina, characterised by a decrease in *Lactobacillus* and an increase in anaerobic bacteria, such as *G. vaginalis, Atopobium* spp., *and Prevotella* spp., which correspond to CST IV-A, IV-B, and IV-C0. Aerobic vaginitis (AV) is a vaginal dysbiosis characterised by the loss of *Lactobacillus* and an increase in aerobes such as *Enterococcus* spp., *Escherichia coli, Staphylococcus* spp., and *Streptococcus* spp. identified by culture and bacterial sequencing (Wang et al., 2020), which may correspond to CST IV-C1, IV-C2, and IV-C4. A healthy metabolite environment positively correlates with the metabolism of lysolipids, phospholipids, glutathione, and glycogen, but negatively correlates with the metabolism of biogenic amines, lysine, and histidine, and is characterised by high lactic acid levels. Both *L. crispatus* and *L. gasseri* produce L-type and D-type lactic acids, *L. jensenii* only produces D-type lactate, and *L. iners* only produces L-type lactate (Witkin et al., 2013). *L. iners* also secretes the cholesterol-dependent cytotoxin, haemolysin. This explains why the dominant vaginal bacteria in healthy women are mostly *L. crispatus*, followed by *L. gasseri* and *L. jensenii*, while *L. iners* is often associated with pathogenic bacteria, for instance, *G. vaginalis*. Metabolites associated with BV positively correlate with the metabolism of biogenic amines, lysine, and histidine; however, they negatively correlate with lipid-, glutathione-, and glycogen metabolism. The levels of biogenic amines (putrescine, cadaverine and trimethylamine) and short-chain fatty acids (SCFAs) (especially acetate, butyrate and formate) in BV are high, while the levels of some amino acids

(tyrosine and glutamate) in BV are low (Srinivasan et al., 2015; Vitali et al., 2015; Watson and Reid, 2018; Borgogna et al., 2020). The metabolite signature of AV is less characterised than that of BV. A study on premature rupture of membranes (PROM) patients shows that the glycolytic metabolite GalNAc (N-acetylgalactosamine) and sucrose negatively correlate with *Streptococcus, Chlamydia, Prevotella*, *Staphylococcus*, *Mycobacterium*, and *Enterobacter* genera, which supports the reduction of lactic acid (Liu et al., 2021b). In addition, *Streptococcus* spp. dominated communities contain slightly higher acetate levels (Gajer et al., 2012).

Immune response induced by cervicovaginal microbiota on cervix

The effect of cervicovaginal bacteria on the cervix is less pronounced than on the vagina. Cervical epithelial cells and immune cells recognize and sense pathogens through pattern recognition receptors (PRRs). Cell-surface PRRs toll-like receptors (TLRs) 1,2,4,5,6 and 3,7,9 (Aflatoonian and Fazeli, 2008; Benjelloun et al., 2020) and intracellular cytosolic pathogen sensing receptors Nod-like receptors (NLRs), NOD1, NOD2, RIG-1, and MDA5 (Ghosh et al., 2013) have been found in the cervix mucosa. TLR1, TLR2, and TLR4-6 detect microbial ligands, TLR2 and TLR4 can recognize Gram-positive and Gram-negative bacteria, respectively, and regulate the release of downstream cytokines. NOD1 and NOD2 can recognize intracellular PAMPs that can enter the cell through phagocytosis or membrane pores (Anton et al., 2022). BV and associated pathogens, such as *Prevotella* and *Gardnerella*, have been shown that associated with the expressions of TLRs and NLRs, especially TLR2/4 (Anahtar et al., 2015; Anton et al., 2022; Dong et al., 2022; Gerson et al., 2022). BV-associated bacteria can induce immune response of cervical cells through the TLR2/4–activated signalling pathways (Zariffard et al., 2005; Anton et al., 2022). In addition, due to *G. vaginalis* can trigger the NLRP3 infammasome in macrophages and monocytes (Vick et al., 2014; Xiang et al., 2021), it is possible that NOD signalling may be another passway of G. vaginalis-mediated infammation in cervicovaginal epithelial cells (Anton et al., 2022).

Clinical studies of the relationship between cervical cytokines and CVM sequencing are listed in Table 1. Most studies show that the BV-associated microbiome (CST IV) and elevated microbiota diversity

TABLE 1 The clinical studies of the relationship between cervical cytokines and CVM sequencing

Author (year)	Population	Ethnicity	Sample	Method	Findings
Gustin et al, (2022)	BV N=28	NS	Vaginal swabs, CVL	16S V3-4	Shift from CT4 to CT2 possessing significantly higher levels of SLPI, GROa, and MIP3a and significantly lower levels of ICAM-1; Highly diverse microbiota are associated with the enhanced resilience of bacterial vaginosis to standard metronidazole treatment; CD4+ cells from the lamina propria were significantly higher in CT4
Lo´pez-Filloy et al, (2022)	Symptomatic cervical ectopy N=156	NS	Cervical secreations	16S V4	In HPV+ and cervical ectopy patients, bacterial diversity correlates with both IL-1βand IL-22 HPV+ women showed a significant decreased in *Lactobacilli* and increase in anaerobes such as *Sneathia, Pseudomonas, Megasphaera, Atopobium, Shuttleworthia, Prevotella*, and *Clostridium*, IL-21 and CXCL9 were significantly upregulated in HPV-positive
Kawahara et al., (2021)	CIN recieved surgery N=28 CIN under observation N=13	NS	Cervical secreations	16S V3-4	IL-1β and TNF-α were signifcantly increased with the presence of anaerobics microbiota; TNF-α, IL-10 and RANTES were inversely correlated with *L. crispatus*
Serebrenik et al., (2021)	Bronx N = 20 Thika N = 18	Mainly Black	CVL, Cervical tissue	16S V3-4 RNA-seq	BV patients who respond to metronidazole treatment had a increase of CXCL-9, CXCL-10, and SLPI and a decrease in IL-1α and IL-1β compared with treatment failure
Balle et al (2020)	130 females adolescent before and after contraceptive use	South African	Vaginal swab cervical secreations	16S V4	Cytokines were positely associated with the following aerobes. IL-17 (*S.anginosis,A.minutum, Mycoplasmataceae*), IL-6 (*peptococcus, Moryella*), IL-1b (*P.rnelaninogenia, Mycoplasma hominis,A.prevoii*), IL-21(*P.timonensis, P.rrica*), IL-23 (*S.sanguinus, Mycoplasmataceae*). The abundances of *L. crispatus* and *L. iners* were negatively correlated with the concentrations of several cytokines. The inflammation-high group had significantly higher alpha diversity compared to the low group and was more likely to be CST IV
Joag et al., (2019)	BV N=45	Keneya	Vaginal swabs, endocervical cytobrush	16S V3-4	BV treatment reduced genital CD4+ T-cell HIV susceptibility and reduced IL-1α/β levels, However, BV resolution and the concomitant colonization by *L. iners* substantially increased several genital chemokines associated with HIV acquisition, including IP-10, MIP-3α
Łaniewski et al. (2018)	HPV-negative controls N = 20, HPV-positive controls N= 31, Low-grade dysplasia N= 12,	Mix	Vaginal swab CVL	16S V4	Sneathia presence VMB associated with IL-1b, IL-36r, TNFa, IL-8,IL-10,MIP1a, TNFb,MIP-1b, RANTES, SCD40L,IL-10,

(Continued)

TABLE 1 Continued

Author (year)	Population	Ethnicity	Sample	Method	Findings
	High-grade dysplasia N= 27 Invasive cervical carcinoma N = 10				

BV, bacterial vaginosis; CVL, cervicovaginal lavage; CST, community state types; KRST, Kenya, Rwanda, South Africa and Tanzania; CT, cerival type; CVM, cervicovaginal microbiota. NS, Not specifically.

are related to an increase cervical IL-1α and IL-1β, and to a lesser extent granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-10 (Mitchell and Marrazzo, 2014; Shannon et al., 2017), which was similarly with vagina (Shannon et al., 2017). The *L. iners* dominant microbiome is associated with increased IP-10 and MIG compared with CST I/II (Anahtar et al., 2015; Shannon et al., 2017). Clinical data of IL-6, IL-8, and IL-10 cytokines and chemokines in the BV-associated microbiome are inconsistent in the vagina and cervix (Mitchell and Marrazzo, 2014). Discrepancies in immune molecules may be attributed to the diversity of microbes and hosts (Łaniewski and Herbst-Kralovetz, 2021). Several studies have explored the role of a single bacterium in epithelial immunometabolic characteristics using a 3D cervical epithelial model. Among the common vaginal bacteria, *Lactobacillus* (especially *L. crispatus*) usually plays a protective role in immunomodulation, *G. vaginalis* or *Prevotella bivia* usually induces a relatively low inflammatory response, whereas *A. vaginae* and *Sneathia amnii* elicit more robust cytokine responses (Eade et al., 2012; Gardner et al., 2020; Łaniewski and Herbst-Kralovetz, 2021). L. crispatus did not induce an inflammatory response or alter the epithelial barrier integrity when cocultured with 3D cervical models. P. bivia and G. vaginalis strains only induce IL-6, TNF-α, IL-1α, and MMP-9, whereas A. vaginae and S. amnii induce multiple proinflammatory molecules, including IL-6, IL-8, IP-10, MCP-1, MIP-3α, RANTES, MMP-10, MMP-1, and sFas. Polymicrobial infection with four BV-associated bacteria leads to a mixed profile and extra activation of IL-1β, a critical cytokine usually raised in women with BV (Łaniewski and Herbst-Kralovetz, 2021). Other BVAB such as Eggerthella sp. only causes an increase in IL-1α; Mobiluncus mulieris increases IL-1α, IL-6, IL-8, MCP-1, and TNF-α; while Megasphaera micronuciformis increases IL-1α, IL-1β, IL-1RA, TNF-α, IL-6, and sFasL (McKenzie et al., 2021). Cervical epithelial cells (especially endocervical epithelial cells) exhibit a more robust immune response than vaginal cells after stimulation by BVAB bacteria. The cervix and the transformation zone of the upper and lower reproductive tracts has a higher ability to generate inflammatory responses than that of the vagina, thus guaranteeing the relative sterility of the upper reproductive tract (Eade et al., 2012).

Research on common cervicovaginal bacteria in immune cells has mainly focused on DC cells, macrophages, and T cell recruitment and differentiation. The effect of *G. vaginalis* on DC cell stimulation and the inflammatory response is not obvious. *G. vaginalis* and its supernatants induce THP-1 macrophages to differentiate into the M1 phenotype which is involved in defence against bacterial infections, elevated reactive oxygen species (ROS) levels, and stimulation of the NF-κB/STAT1 pathway (Liu et al., 2021a). This causes THP-1 cell pyroptosis by promoting the formation of the NLRP3 inflammasome, resulting in cytokine secretion. Other BVABs such as *Megasphaera* elsdenii and Prevotella timonensis significantly promote the maturation of DC cells, the differentiation of T cells into the pro-inflammatory Th1 type, and the increase of IL-1β, IL-6, IL-8, IL-12p40, and TNF-α (van Teijlingen et al., 2020). The increase in Th1 pro-inflammatory cytokines Il-1β, IL-6, and IL-8 recruits Th1 and Th17 pro-inflammatory $CD4^+$T cells, effector memory $CD8^+$T cells, and leucocytes (Deruaz and Luster, 2015). In contrast, vaginal Lactobacillus inhibits the pro- or inflammatory response of epithelial or immune cells (Jan et al., 2012) and promotes M2 macrophages polarisation which is involved in tissue repair and wound healing (Lin et al., 2021), promotes the differentiation of $CD4^+$T cells toward immunosuppressed Treg cells (Jang et al., 2017), but has no effect on DC maturation (van Teijlingen et al., 2020).

Immune response induced by cervicovaginal metabolites on cervix

The most representative metabolites in the cervicovaginal tract are lactic acid and SCFAs. Lactic acid and other products such as extracellular polysaccharides (EPS) exhibit anti-inflammatory effects on both cervical epithelial and immune cells, while the effect of SCFAs is not well understood.

EPS secreted by vaginal L. gasseri strains G10 and H15 inhibit the inflammatory factor TNF-α and promote the increase in the anti-inflammatory factor IL-10 (Sungur et al., 2017). Coculture of protonated L/D lactate with cervical epithelial cells results in a decrease in IL-6 and IL-8, and an increase in IL-1RA and IL-1β (Hearps et al., 2017). IL-1RA is expressed more highly than IL-1β, and is an antagonist of IL-1β, which inhibits its pro-inflammatory effect (Hearps et al., 2017). Delgado-Diaz et al. (Delgado-Diaz et al., 2019) simulated the organic acid composition of healthy or BV women (normal: 33 mM L- lactic acid, 4 mM acetate, 1 mM succinate, pH 3.9; BV: 100 mM acetate, 20 mM succinate, 4 mM propionate, 4 mM butyrate, pH 7). Normal organic acid dampening TLR-elicited stimulation of IP-10 MIP-3α, IL-6 of cervicovaginal epithelial cells. Regarding the effect of lactic acid on immune cells, tumour-related studies demonstrate that lactic acid has an immunosuppressive role by inhibiting the differentiation of monocytes and T cells, activity of cytotoxic $CD8^+$T cells (CTLs), maturation of DC cells, and promoting polarisation of macrophages towards the M2 type (Ma et al., 2020).

The effect of SCFAs is studied more fully in the gut than in the female reproductive tract. SCFAs act as an energy source and immune modulator of the intestinal cell. Most studies show that SCFAs

(especially butyrate) restore intestinal barrier function in inflammatory conditions by exhibiting anti-inflammatory effects in intestinal mucosa and inducing tight junction protein expression (Parada et al., 2019), although some studies show contradictory results. For instance, SCFA (acetic, butyric, and propionic acids) cocultured with peripheral blood mononuclear cells (PBMCs) and neutrophils significantly enhance TLR2 and TLR7 and induces IL-8 and TNF-α in a time- and dose-dependent manner (Mirmonsef et al., 2012). Propionate and butyrate also increased in vitro transmigration of neutrophils (Vinolo et al., 2009). SCFAs induces both effector and regulatory T cells by suppressing histone deacetylases and regulating the mTOR-S6K pathway (Arpaia et al., 2013; Park et al., 2015). The difference depends on the type, concentration and pH of the SCFAs and cell type. In the female reproductive tract aspect, BV organic acids (especially acetic and butyric acids) enhance the secretion of TNF-α after TLR1/2/3 stimulation of cervicovaginal epithelial cells but inhibit the production of IL-6, RANTES, and IP-10 (Delgado-Diaz et al., 2019). The exact role of SCFA in the reproductive tract immune cells remains to be elucidated.

The effect of cervicovaginal dysbiosis on epithelial barrier disruption

Clinical proteomic and transcriptional studies show that CVM is associated with the disruption of the cervical epithelial barrier (Table 2). Dysbiosis of vaginal flora may disrupt the epithelial barrier by inducing an inflammatory response.

Borgdorff et al. divided vaginal microbiota composition into four groups according to bacterial diversity: group 1 (*L. crispatus*-dominated), group 2 (*L. iners*-dominated), group 3 (moderate dysbiosis, mixture of BV), and group 4 (severe dysbiosis, all BV-

TABLE 2 Clinical studies of cervicovaginal proteome and RNA-sequencing.

Author (year)	Population	Ethnicity	Sample	Method	Findings
Delgado-Diaz et al. (2022)	Nugent-BV N=56 Non-BV N=57	Black	Cervicovaginal swab	16s rRNA Host proteome	Women with L. iners dominated flora contain primarily bacterial L-LDH, women with L. crispatus-dominated microbiome contain both L- and D-LDH Between high L-LDH and low L-LDH, 31 of different proteins having epithelial barrier function related gene ontologies include tight junction protein 1, keratin 8, dermokine
Serebrenik et al. (2021)	BV N=38 before and after metronidazole treatment	NS	Vaginal swabs CVL Ectocervical tissue	16s V3-4 RNA-sequencing	Responders vs nonresponders: lower levels of CXCL9 in cervicovaginal lavage on day 0. Concentrations of CXCL9, CXCL10, and monocyte chemoattractant protein 1 increased signifcantly between day 0 and day 35
Farr Zuend et al. (2020)	Healthy pregnant N = 23 Non-pregnant N= 25	NS	CVL	Human and Microbial proteome	Microbial proteome :L-lactate dehydrogenase, Glyceraldehyde-3-phophospate dehydrogenase type I, pyruvate kinase, and phosphoglycerate kinase were primarily driving the variance within the LD group, while transaldolase was responsible for the variation in the nLD group
Ferreira et al. (2018)	BV N=29 Health controls N=29	MIX	CVL	Human proteome	BV CVL: Neutrophil elastase, kaliocin-1, neutrophil defensin-1, Ig lambda-2 chain C regions, and protein S100-A7↑. Normal CVL: immune modulation and epidermis development leukocyte elastase inhibitor, serpin B4, cluster of proline-rich protein 2A, smallproline-richprotein3, cluster of cornifin-B, cellular retinoic acid binding protein 2, and fatty acid binding protein↑.
Bradley et al. (2018)	Lactobacilli dominant group N=32 Non-Lactobacilli dominant group N=15	NS	Cervicovaginal swab sampled at different menstrual cycle phase	16s V3-4 Human proteome	The luteal and follicular phases associate with higher activation of neutrophil/leukocyte pathways and cell migration pathways, Ovulatory phase exhibits increased of antimicrobial and wound-healing pathways and reduced inflammatory cytokines. Women without Lactobacillus showed an amplification of hormone associated variation such as the decrease in the epithelial barrier protein RPTN during the luteal phase
Borgdorff et al. (2016)	L. crispatus-dominated (group1) N=7 L. iners-dominated (group2) N=11 Moderate dysbiosis (group3) N=14 Severe dysbiosis (group 4) N=18	NS	Cervical spatulas and cytobrushes, CVL	Human proteome	From group1 to group4: AMP and cytokines(cystatin A,lysozyme C, ubiquitin ↓;histones, psoriasin, calprotectin↑, IL-36a, C5, MIF↑ Mucus (MUC5B、MUC5AC↑) Cytoskeleton alterations(KRT4、5、6A↓; LDHA, LDHB↑) Humoral immune response(IGHG1 and IGHG2↓)

(Continued)

TABLE 2 Continued

Author (year)	Population	Ethnicity	Sample	Method	Findings
Zevin et al. (2016)	cohort 1 n=10 coehort 2 n=31	Black(n = 27) Caucasian (n = 3) Asian (n = 2) Hispanic (n = 1).	Vaginal swabs CVL	16s V3-V4 Human and microbial proteome	Gardnerella vaginalis dominat women associated with host epithelial barrier disruption and enhanced immune activation; Lactobacillus species dominat women experised host mucosal proteins important for maintaining epithelial integrity.
Arnold et al. (2016)	Elevated inflammatory cytokines N=28 Controls N=68	Black	Endocervical cytobrush, CVL	Human proteome	Elevated inflammatory cytokines were associated with proteases, cell motility, and actin cytoskeletal pathways, whereas protease inhibitor, epidermal cell differentiation, and cornified envelope pathways were decreased.
Anahtar et al. (2015)	CT1 N=8 CT2 N=27 CT3 N=28 CT4 N=31	Black	Ectocervical vaginal swabs, CVL, endocervical cytobrush	16S V4 Whole-genome shotgun sequencing RNA-seq	The genes upregulated in APC of CT4 were enriched in NF-kB, Toll-like receptor (TLR), NOD-like receptor, TNF-a signaling pathways and many pro-inflammatory cytokine genes compared with CT1 and CT2
Cruciani et al. (2013)	Health N=40 BV N=39	NS	CVL	Human proteome	BV vs. Healthy: The majority of immunoglobulins↑, mucus (Mucin-5B↑) Epidermis development and keratinization (cadherin-1, Desmocollin-2, Desmoglein-3↑; Keratin5↓) Cytoskeleton remodelling, blood coagulation and complement activation were the most enriched passways

BV, bacterial vaginosis; CVL, cervicovaginal larvage; CT, cerival type. NS, Not specifically. ↑, increase; ↓, decrease.

positive). An increase in the vaginal microbiota group causes proteomic changes including cytoskeleton alterations (increasing actin-organising proteins, decreasing keratins and cornified envelope proteins), increasing lactate dehydrogenase (LDH) A/B as markers of cell death, proinflammatory cytokines, and proteolytic activity, together with decreasing immunoglobulin G1/2, AMP imbalances, and mucus alterations (increasing MUC5B and 5AC) (Borgdorff et al., 2016). Additionally, the cervicovaginal microbiome influences certain menstrual cycle-dependent changes to the cervical epithelial barrier, where hormone-associated reduction of the epithelial barrier protein RPTN is amplified in women without *Lactobacilli* during the luteal phase (Bradley et al., 2018). Other proteome studies demonstrate that CST III/IV or BV have differentially expressed proteins involved in the cytoskeleton, keratinisation alteration, epidermis development, and immune response compared with healthy women (Cruciani et al., 2013; Zevin et al., 2016; Ferreira et al., 2018). The inflammatory response contributes to the disruption of the cervicovaginal epithelium. Arnold et al. found elevated cytokine expression is positively associated with neutrophil proteases (MMP-9 and MMP-8), reduced antiproteases (SPINK5, SPINK7, SLPI), and altered cytoskeleton, epithelial differentiation, and keratinisation pathways. IL-1β, MIP-3α, and IL-8 display the strongest correlations with MMP-8 and MMP-9, indicating reduced barrier integrity (Mohammadi et al., 2022). IL-1β activates p38 and JNK signalling, leading to decreased tight junctions and disruption of mammary epithelial intergrity (Kobayashi et al., 2021). TNF-α and lipopolysaccharide (LPS) increase apoptosis, necrosis, and senescence of cervical epithelial cells (Tantengco et al., 2021a). The dysbiotic microbiome induces an inflammatory response which contributes to the disruption of cervicovaginal epithelial cells.

In addition, CVM and its metabolites directly influence the epithelial barrier by inducing oxidative stress, altering miRNA expression, and promoting cell cycle arrest and apoptosis. BV-associated bacteria induce oxygen stress intermediates when cocultured with a 3D cervical epithelial model which affects the epithelial barrier integrity (Wang et al., 2021b). *P. bivia* and *S. amnii* increase 2-hydroxyglutarate, 2-hydroxybutyrate, and citrulline levels, which are correlated to the activity of inducible nitric oxide synthase (iNOS) (Łaniewski and Herbst-Kralovetz, 2021). *Eggerthella* sp. and *M. mulieris* elevate sphingolipids and 2-hydroxybutyrate and decrease cysteinylglycine and cysteinylglycine disulphide (McKenzie et al., 2021). *Veillonella atypica* and *Veillonella montpellierensis* significantly accumulate histamine. *Fusobacterium gonidiaformans* and *Fusobacterium nucleatum* increase 2-hydroxyglutarate, induce cysteine- and methionine metabolic pathways, pro-inflammatory lipids, and genotoxic hydrogen sulfide (Maarsingh et al., 2022). 2-hydroxyglutarate is a metabolite marker of oxidative stress. The depletion of two intermediates in the glutathione synthesis pathway (cysteinylglycine and cysteinylglycine disulfide) indicates increased glutathione biosynthesis and an increase in ROS levels (McKenzie et al., 2021). The distribution of ROS or oxidative stress pathways in human cervical epithelial cells and cervical stromal cells is linked to sterile inflammation (increased IL-6). This is mediated by p38 mitogen-activated protein kinase activation which promotes cell cycle arrest and cell necrosis death (Tantengco et al., 2021b), and is associated with lipid peroxidation and epithelial tight junction disruptions (Yamamoto et al., 2021). Histamine decreases

tight junctions (ZO-1 in nasal epithelia and E-cadherin in pulmonary epithelia) (Salliss et al., 2021). The increase in sphingolipids also reflects disruption of the epithelial barrier.

BV-associated bacterial supernatants also affect the alteration of epithelial barrier function. Supernatants derived from *G. vaginalis* and *L. iners* increase ectocervical and endocervical cell permeability, cleave E-cadherin, and elevate miR-143 and miR-145 expression, which downregulate cell adhesion genes, JAM-A and FSCN1, decrease cell proliferation, and increase apoptosis (Anton et al., 2017). *M. mulieris* supernatant also increases cell permeability (Dude et al., 2020). In contrast, lactic acid increase the expression of barrier proteins claudin1, claudin4 of cervicovaginal epithelial cells (Delgado-Diaz et al., 2022), *L. crispatus* supernatants also can mitigate the disruption of cervical epithelial barrier induced by LPS or *G. vaginalis* and reversal of *G. vaginalis*-induced increase in miRNA expression (Anton et al., 2018). *G. vaginalis* secretes haemolysin (vaginolysin), a cholesterol-dependent pore-forming toxin that increases the membrane permeability of host cells and causes K^+ efflux, activates caspase-1, and contributes to apoptosis (Muñoz-Planillo et al., 2013; Vick et al., 2014). The increase of sFasL (an apoptosis-related protein) in cervicovaginal lavages of women with vaginal dysbiosis demonstrates the damage and apoptosis of cervicovaginal epithelial cells. Taken together, vaginal dysbiosis bacteria can directly induce epithelial barrier disruption through oxidative stress and miRNA alteration, and promote cell cycle arrest, apoptosis, and necrosis. An indirect effect is also realized by the secretion of harmful metabolites and causing immune disorders (Figure 1).

The effect of cervicovaginal microbiota on mucus and cervical remodeling

Mucus and mucins play a role in protecting mucosal surfaces, protecting the epithelium from contact, and removing intruders by trapping (Hansson, 2020). Mucins provide an attachment site and a nutrient source for some bacteria. For example, isolated vaginal *Lactobacilli* have shown the ability to bind mucin (Leccese Terraf et al., 2014; Ahire et al., 2021). Vaginal *Lactobacilli* contain genes encoding proteins that allow adhesion to mucins, including mucus adhesion promoting protein (MapA), mucus-binding protein (MubI), cell and mucus-binding protein (CmbA), modulator of adhesion and biofilm formation (mabA), and mucus binding factor (MBF) (Leccese Terraf et al., 2014). *Lactobacilli* isolated from the gut contain several mucus adhesion proteins, including mucus-binding protein (MUB), MucBP domain, pili, SIpa, MapA, and EF-Tu. These adhesion proteins include two main patterns: MUB, the MucBP domain, and pili are cell wall-anchored proteins that covalently bond to the cell wall; MapA, EF-Tu, and ATP-binding cassette (ABC) transporters are multifunctional proteins (so-called moonlighting proteins) that, in addition to performing their primary intracellular functions, also act as adhesion molecules (Nishiyama et al., 2016).

BVAB alters the cervical mucus by stimulating mucin production and degrading highly glycosylated proteins. Clinical studies show that the levels of MUC1, MUC4, MUC5AC, MUC5B, and MUC7 are significantly higher in women with BV than in women with normal or intermediate Nugent scores (Borgdorff et al., 2016; Moncla et al., 2016). Bacterial and viral products and TNF-α increase the mRNA expression of MUC1, MUC4, and MUC16 in a 3D endocervical model (Radtke et al., 2012). An increase in MUC expression enhances the ability of epithelia to form a protective barrier, thus promoting bacterial clearance. Sialidase and glycosulfatase levels and activity increase in BV patients compared with normal women (Gipson et al., 1999; Gipson et al., 2001; Wiggins et al., 2002; Ng et al., 2021). *G. vaginalis* strains, *Prevotella* species, and *S. amnii* liberate or consume sialic acids (Łaniewski and Herbst-Kralovetz, 2021). Three sialidases are identified in *G. vaginalis* strains: NanH1 (formerly sialidase A), NanH2, and NanH3. Among these, NanH2 and NanH3 have higher ability against sialic acid substrates in many different molecular contexts, such as 2-3- and 2-6-linked sialic acids, and N- and O-linked sialoglycans found on SIgA and mucin (Robinson et al., 2019). The only Prevotella spp. that contains a gene encoding α-N-acetylglucosaminidase (an enzyme that cleaves O-glycans of type 3 mucins) is P. *timonensis*; consequently, this species has the greatest sialidase activity in this genus (Ilhan et al., 2020).

Vaginal dysbiosis is associated with cervical shortening (Di Paola et al., 2020) and PTB (Gerson et al., 2022). The cervix needs to be extensively remodelled during pregnancy to allow a full term foetus to pass through the birth canal, which is divided into softening, ripening, dilation/labour, and postpartum repair. Both the cervical epithelium and stroma undergo changes during this process. Cervical epithelia proliferate in preparation for parturition and regulate the expression

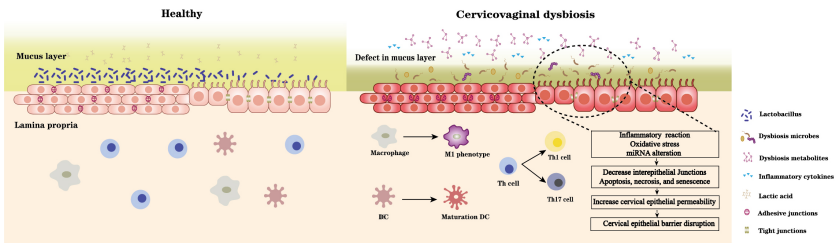

FIGURE 1
A healthy Lactobacillus dominant microbiota and the acidic environment formed by the associated metabolites especially lactic acid maintain cervical epithelial barrier integrity, stabilise the mucosal immune system. In contrast, microbiota dysbiosis and its accompanying changes in microbial metabolites can 1) damage mucus layer; 2) imbalance immune system: promote immune cells differentiate towards proinflammatory type and induce pro- and inflammatory cytokines secretion; 3) disrupt cervical epithelial barrier: induce inflammation reaction, oxidative stress, miRNA alteration of cervical epithelial cell, decrease intracellular junctions, and promote cell cycle arrest, apoptosis, and necrosis. Thus, increase the epithelial permeability and disrupt barrier function.

of aquaporin water channels to maintain fluid balance, epithelial permeability, and a protective barrier. This regulates solute transport through the intracellular junctional complex and protects the cervical stroma and upper reproductive tract against invasion of pathogens (Timmons et al., 2007). Stromal changes include increasing hyaluronan content and collagen solubility (Read et al., 2007), and loosening of the collagen matrix (Yellon, 2019).

The potential mechanisms by which bacterial infections affect cervical remodelling mainly involve metalloproteinases. *G. vaginalis*, *A. vaginae*, *P. bivia*, and *P. asaccharolytica* can induce or secrete MMP-1, 9 and 10, which shows the ability to degrade collagen (type I and IV), gelatin, casein, and fibrinogen (Łaniewski and Herbst-Kralovetz, 2021; McGregor et al., 1986; Łaniewski and Herbst-Kralovetz, 2021; Short et al., 2021; Tantengco et al., 2021a), while *L. crispatus* has no effect on type I collagen, casein, and fibrinogen, which inhibits clot formation (Lithgow et al., 2022). The colonisation of the mouse reproductive tract by *G. vaginalis* increases mucin expression, dispersion of collagen fibres, and alters cervical biomechanical properties which indicates a more rapid cervical remodeling (Sierra et al., 2018). The ability to secrete or induce metalloproteases and degrade collagen highlights their potential to alter reproductive tissue structure and harm human pregnancy through premature cervical remodelling, clotting disruption, and foetal membrane weakening.

The effect of cervicovaginal microbiota on STI infection

CT infection

Chlamydia trachomatis (CT) is the most prevalent sexually transmitted bacterial infection worldwide, with approximately 131 million new cases occurring annually (Kreisel et al., 2021). In some cases, it ascends from the cervix to the uterus and fallopian tubes, leading to severe reproductive pathology (Brunham and Rekart, 2009). It is characterised by an asymptomatic infection and a high reinfection rate. The transmission rates of CT are between 25–40%. Only 50% of women will naturally resolve the CT infection within one year (Morré et al., 2002; Molano et al., 2005). CVM and its products may serve as important cofactors for infection susceptibility, clearance, or reinfection.

BV is the most frequent vaginal infection and is found in 20–48% of CT-infected women (Wiesenfeld et al., 2003; Filardo et al., 2019). A growing number of sequencing studies show the association of cervicovaginal microbiota with CT infection (Table 3). A recent meta-

TABLE 3 The clinical studies of cervicovaginal microbiota sequencing in CT infection women.

Author (year)	Population	Ethnicity	Sample	Method	Findings
Chen et al. (2021)	Tubal infertile women with CT (CT-P) N=9, without CT(CT-N) N=8 Healthy women without CT (CT-C) N=7	Asian	Cervical swabs	16S V3-V4	CT-P: The abundances of most *Lactobacillus*, including *L. crispatus, L. gasseri, L. jensenii, L. reuteri*, and *L. aviaries*↓; *L. iners* and *Veillonellaceae bacterium KA00182*↑; IFN-g and IL-10↓
Mott et al. (2021)	CT naturally cleared population N=13 CT persisted population N=42	Mainly black	Cervical cytobrush	16S V4	Vaginal microbiota may be a stronger predictor of genital cytokine signatures, which may more accurately explain differences in CT clearance. IFN-g *in vivo* were significantly below those needed to clear CT infections in *in vitro* models, also were not significantly different in CT clearers and non-clearers. IFN-g may act together with other factors *in vivo*.
Chiu et al. (2021)	CT positive patients N=22 Healthy controls N= 36	Asian	Vaginal swabs	16S V3–V4	In CT-positive patients, the vaginal microbiota was dominated by *L. iners*, and the relative abundance of *Gardnerella vaginalis* (12.46%) was also higher than that in TV-positive and healthy controls
Raimondi et al. (2021)	CT positive women N=10 Healthy controls N=16	Caucasian	Vaginal and anal swabs	16S V3–V4	In CT-positive patients, the abandance of *Lactobacilli* were reduced and with higher evenness; dysbiosis-associated bacteria (e.g., *Sneathia, Parvimonas, Megasphaera, Ezakiella* spp) were increased
Tamarelle et al. (2020)	CT positive women N=149 Healthy controls N=99 Test every 3 months, a total 3 times	Mainly black	Vaginal swabs	16S V3–V4	CT+ women: was dominated by *Lactobacillus iners*(CST III) or a diverse of bacterial vaginosis–associated bacteria (CST IV). *L. iners*–dominated communities were most common after azithromycin treatment, suggesting that the impact of antibiotic treatment on the vaginal microbiota could favor reinfections.
Filardo et al. (2019)	CT positive women N=42	NS	Cervical swabs	16S V4	CT+ women: cervical microbiota categorized CST-IV were dominant. A specific bacterial network was identified as a potential biomarker, characterized by

(Continued)

TABLE 3 Continued

Author (year)	Population	Ethnicity	Sample	Method	Findings
	Healthy controls N=103				*G.vaginalis, P. amnii, P. buccalis, P. timonensis, A. christensenii*, and *V. guangxiensis*. Cervical lactoferrin, IL-6, IL-1, IFN-β,and IFN- α ↑, IFN- γ↓
Cheong et al. (2019)	CT positive women N=40 Healthy controls N=30	Asian	Cervical swabs	16S V3–V4	CT+ women: mostly strict and facultative anaerobes such as *Streptococcus, Megasphaera, Prevotella, and Veillonella genera*↑
Parolin et al. (2018)	CT positive women N=20, Healthy controls N=22 BV N=30	Caucasian	Vaginal swabs	Vaginal Array	CT+ women: *L. iners*↑, Asymptomatic CT-positive patients are more likely to have *L. crispatus* than women with CT-correlated symptoms, 4-aminobutyrate were lower in asymptomatic than symptomatic CT women
Balle et al. (2018)	CT positive women N=30 Healthy controls N=42	NS	Vaginal and cervical swabs	16S V4	CT+ women: more likely to have community type dominated by diverse anaerobic bacteria (C1) or L. iners (C3)
Di Pietro et al. (2018)	HPV/CT positive women N=30 Healthy controls N=43				HPV/CT co-infected women had a higher microbial diversity, Aerococcus christensenii was associated with C. trachomatis infection.
Filardo et al. (2017)	CT positive women N=10 Healthy controls N=35	NS	Cervical swabs	16S V3–V4	CT+ women: an overall decrease in Lactobacillus spp, diversity and anaerobes ↑
van der Veer et al. (2017)	CT positive women N=52, Healthy controls N=41	Mainly European	Cervical and/or vaginal swab	16S V3–V4	Diverse anaerobic CVM and L. iners–dominated CVM were independently associated with CT infection compared with women with L. crispatus–dominated CVM

CT, Chlamydia trachomatis. NS, Not specifically. ↑, increase; ↓, decrease.

analysis reveals a trend towards a positive association between low *Lactobacilli* microbiota and HPV and CT infection, suggesting a potential protective role of high *Lactobacilli* microbiota (Tamarelle et al., 2019). *Lactobacilli* including *L. crispatus*, *L. gasseri*, *L. jensenii, L. reuteri*, and *L. aviarius* are reduced in both the cervical and vaginal microbiota of CT-positive women (Filardo et al., 2017). Asymptomatic CT-positive patients are more likely to have *L. crispatus* than women with CT-correlated symptoms. In contrast, BVAB, *L. iners*, and increased species richness and diversity are associated with CT infections (Filardo et al., 2017). A specific bacterial network characterised by BV-associated bacteria include, *G. vaginalis, Prevotella amnii, Prevotella buccalis, P. timonensis, Aerococcus christensenii*, and *Vatica guangxiensis* is a potential biomarker of CT (Filardo et al., 2019). In addition, *L. iners*-dominated CVM is an independent risk factor for CT infection compared to *L. crispatus*-dominant CVM (van der Veer et al., 2017; van Houdt et al., 2018).

Some studies have explored the mechanisms of CVM in CT treatment and clearance. Mott et al. (Mott et al., 2021) evaluated the potential impact of BV and metronidazole treatment on CT clearance and found that CT clearers were more likely to have a *Lactobacillus*-dominant vaginal microbiota after metronidazole treatment compared to non-clearers. The diverse anaerobe dominant group has higher IL-1α and IL-1β levels, while the *L. crispatus* or *L. iners* dominant group has higher IL-6 and IP-10 levels, which is consistent with CT clearers. IP-10 can result in a rapid influx of T cells which may aid CT clearance. IP-10 can be abrogated in endocervical epithelial cell secretion after CT infection. Cervicovaginal dysbiosis and BV treatment can drive cytokine changes, which may modulate CT clearance. Although *L. iners* dominant flora and its relative cytokine IP10 may help CT clearance. *L. iners* and *G. vaginalis* have a higher level of resistance to azithromycin and, thus could be selected for post-treatment, whereas sensitive *Lactobacillus* spp. are diminished (Tamarelle et al., 2020). The instability of the *L. iners* dominant flora, its ease of conversion to BV-associated flora, and the potential risk factors for CT infection suggest that the risk of STI in women may not be reduced after antibiotic treatment.

IFN-γ induced persistence of CT in epithelial cells is another issue through which CVM can impact the pathogenesis of CT. CT is characterized by two distinct forms, including extracellular infectious basic bodies (EBs) and intracellular dividing reticulums (RBs). CT is a tryptophan auxotroph. IFN-γ produced by infiltrating T and NK cells induces the activation of the enzyme IDO1, which depletes the tryptophan into kynurenine. Lacking tryptophan can arrest CT in intracellular RBs, resulting in morphologically aberrant, viable but non-cultivable, persistent growth form (Beatty et al., 1994; Aiyar et al., 2014; Ziklo et al., 2019). CT contain a tryptophan synthase operon (trpA, trpB and trpR genes) that can produce tryptophan from indole. *Lactobacillus* spp. do not produce indole, however, many *Prevotella* spp. can produce indole and promotes CT infection in the presence of IFN-γ (Ziklo et al., 2016). Besides *Prevotella* spp., *Porphyromonas asaccharolytica, Propionibacterium acnes, Fusobacterium nucleatum, Faecalibacterium prausnitzii, Enterococcus faecalis, Peptoniphilus harei*, and *Escherichia coli* are also found the ability of indole-producing (Ziklo et al., 2018). In

addition to tryptophan depletion, a recent study found that IFN-γ also can down regulate c-Myc, the key regulator of host cell metabolism, resulting in the reduction of TCA cycle intermediates and nucleotides to prevent chlamydial replication and promoting persistence (Rajeeve et al., 2020; Vollmuth et al., 2022). Constitutive expression of c-Myc can rescue CT from persistence induced by IFN-γ. Dong et al. (Dong et al., 2022) proved that the abundance of *Prevotella* correlates with the expression of cervical TLR4, NF-κB, C-myc, and hTERT genes. In contrast, *Lactobacillus* abundance showed negative correlations with these genes. Whether CVM can impact IFN-γ involved CT pathogenesis and persistence through C-Myc remain to be verified.

Most experimental studies have concentrated on the protective effect of *Lactobacillus* spp. towards CT infections. CT replicates only intracellularly; therefore, attachment and entry are essential in CT infection and pathogenesis. (Mastromarino et al., 2014). *L. brevis* and *L. salivarius* can co-aggregate with CT and compete with HeLa cells, thereby reducing the proportion of EB and inhibiting EB absorption. The intracellular multiplication of CT are inhibited by *L. brevis* and *L. salivarius* (independent of the pH) (Mastromarino et al., 2014). Vaginal *Lactobacilli* inhibit CT adhesion and infectivity in human epithelial cells and macrophages (Rizzo et al., 2015; Parolin et al., 2018). Incubation of HeLa cells with *L. crispatus* BC5 cells reduces polar lipids and α5β1 integrin subunit exposure in the epithelial plasma membrane. The interaction of CT with the β1 integrin subunit of host epithelial cells is a mechanism for EB binding, invasion, and signalling (Stallmann and Hegemann, 2016).

Lactobacillus supernatants inhibit CT infectivity; *L. crispatus* is the most potent while *L. iners* is the least effective (Edwards et al., 2019; Chen et al., 2021). The supernatants of D-lactate-producing Lactobacillus spp.(*L. crispatus* and *L. jensenii*) modulate the expression of multiple genes related to cell proliferation including decreasing miR-193b and histone deacetylase 4 (HDAC4) which are required for CT-induced proliferation during infection. HDACs can repress gene transcription of cyclin-dependent kinase inhibitor 1A (CDKN1A), which in turn inhibits the activity of cyclin-dependent kinase 4 (CDK4) and thus the cell cycle. Downregulation of HDAC4 by *L. crispatus* and *L. jensenii* supernatants led to an increase of CDKN1A and lower host cell proliferation. Therefore, these extracts help reduce susceptibility to CT infection (Edwards et al., 2019). D-lactic acid is the most potent metabolite and could compensate for the inhibitory effect of *L. iners* on CT detection. Lactoferrin is another metabolite that inhibits CT entry into host cells and downregulates IL-6/IL-8 synthesis. Its inhibitory role is proven by the administration to pregnant women asymptomatically infected with CT (Sessa et al., 2017). *Lactobacilli* and their products also modulate the host inflammatory reaction after CT infection. For instance, *L. crispatus* and its supernatant reduce production of IL-6, IL-8, and TNF-α and increase IL-10 production in CT-infected HeLa and J774 cells (Rizzo et al., 2015). *Lactobacilli* mixtures significantly reduce CT-induced cytokine production (TNF-α, IFN-γ, and IL-1β) in the mouse vagina and the severity of hydrosalpinx, oviduct inflammation, and dilatation (Chen et al., 2022). *L. crispatus* reduces the levels of pro-inflammatory cytokines to reduce inflammatory symptoms, which may explain why asymptomatic CT-positive patients are more likely to have *L. crispatus* than women with CT-correlated symptoms.

HPV infection and relative disease

Most existing epidemiological studies support a positive association between vaginal infection and HPV acquisition, persistence, and cervical intraepithelial neoplasia (CIN) progression. Many case-control-, cross-sectional-, longitudinal-, and meta-analysis studies demonstrate that BV is the most relevant vaginal infection. A meta-analysis including 13 studies demonstrated that the factors related to HPV infection are BV, vulvovaginal candidiasis (VVC), CT, and ureaplasma urealyticum (UU), with BV also related to CIN. Another study also showed that moderate-to-severe AV are correlated with CIN (Plisko et al., 2021). A network meta-analysis of the microbiome including 11 longitudinal and cross-sectional studies showed that a low *Lactobacilli*-dominated CST and greater microbiota diversity are strongly associated with HPV compared with *L. crispatus*. *L. iners* also showed higher odds of HPV infection than *L. crispatus* (Norenhag et al., 2020; Usyk et al., 2020; Zhang et al., 2022). Women who acquire HPV16 are more likely to transition between microbial communities than women without a previous history of HPV infection (Moscicki et al., 2020). Taken together, vaginal dysbiosis associated with CST, higher diversity, and unstable transition properties are associated with HPV infection and progression (Supplementary Table 1). Most studies have shown the harmful role of BVAB in HPV infection and related diseases, including *Gardnerella, Prevotella, Atopobium*, *Sneathia*, and *Mycoplasma*, although a few studies show inconsistent results, which is the same as that of *L. iners*. Another longitudinal cohort study found that depletion of *Lactobacillus* spp. and the existence of specific anaerobic bacteria (including *Megasphaera* spp., *P. timonensis and G. vaginalis*) are associated with persistence and slower regression of CIN2 (Mitra et al., 2020).

As previously discussed, BV-associated bacteria can induce cervical inflammation, influence local immunity, and disrupt the epithelial barrier. This impact may contribute to HPV infection and disease progression. HPV infection also promotes cervicovaginal microbiota imbalance by influencing the expression of mucosal host defence peptides. Lebeau et al. demonstrated that HPV E7 oncoprotein substantially inhibits host defence peptide expression including HβD1, 2, 4, HD-5/6, SLPI, S100A7, and elafin by interacting with NF-κB and Wnt/β-catenin signalling pathways. All these peptides have antimicrobial activity against *G. vaginalis* except HβD1. Meanwhile, S100A7 and elafins expressed by the cervical/vaginal squamous mucosa can be cleaved, internalised, and used as an amino acid source by *Lactobacilli*, which leads to its survival. Consequently, the amino acid source supporting the survival of *Lactobacillus* species is considerably decreased, promoting an unbalanced cervicovaginal flora (Lebeau et al., 2022). This is consistent with work showing that HPV infections result in an increase in vaginal bacterial richness and diversity and a decrease in the percentage of *Lactobacilli*; this causes a shift from CST III to CST IV, despite the status of CINs (Chen et al., 2020).

CVM can affect HPV infection by modulating the inflammatory environment. The antiviral-specific immune response to HPV infection needs the cooperation of $CD4^+$T helper cells and cytotoxic $CD8^+$T cells (CTLs). High levels of IFN-γ secreted by Th1 cells potentiate the cytotoxic activity of CTLs and target pathogen-infected cells. IFN-γ also promotes the expression of intracellular

antiviral genes that block viral replication (Hickey et al., 2011). In contrast, Th17 and IL-17 are associated with an enhanced cervical immune response during HPV infection, result in the progression of cervical lesions (Sahu and Khare, 2021). The number of Th17 cells in the blood and the level of IL-17 in tissue homogenates of the cervix increases at different stages of cervical lesions (Xue et al., 2018). Nicolò et al. stimulated SiHa and CaSki cells with heat-inactivated bacteria to explore the relevant changes in cytokines: Lactobacilli (especially *L. gasseri* and *L. jensenii*) significantly induce IFN-γ levels, while L. ines and *G. vaginalis* increase IL-17 in addition to IFN-γ (Nicolò et al., 2021). An increase in IL-17 and IL-17-inducing cytokines (IL-23 and IL-1β) are observed in the cervicovaginal lavage obtained from women with CST-IV-dominated microbiota (Gosmann et al., 2017). Besides *G. vaginalis*, *Streptococcus anginosus*, *Staphylococcus*, and *Mycoplasmataceae* correlated with an increase in IL-17, cervical lesions, and cancer (Fan et al., 2021b; Manzanares-Leal et al., 2022). In contrast, dominant flora composed of *L. gasseri* and *L. jensenii* are associated with HPV clearance and the prevention of low-grade cervical dysplasia progression (Brotman et al., 2014; Mitra et al., 2015). In addition to IL-17, continuous low IL-1β/IP-10 (BV cytokine signature), predicts HR-HPV clearance, and an elevated TNF-α/MIP-1β signature is associated with CIN2+ progression (Usyk et al., 2022).

Regarding the role of cervicovaginal organic acids in HPV and related diseases, many experts have explored whether *Lactobacilli* and their suppernant have a protective effect against disease. *L. crispatus* culture supernatants promote healing of damaged vaginal epithelium (Takada et al., 2018). *G. vaginalis* supernatants inhibit wound healing of cervical cell monolayers compared to *L. iners* (Zevin et al., 2016). *L. crispatus*, *L. rhamnosus*, *L. gasseri*, and *L. jensenii* supernatants reduce the expression of autophagy genes ATG14, BECN1, and cyclin A, cyclin-dependent kinase 2 (CDK2), and HPV E6 and E7 to inhibit cervical cancer cells activity (Motevaseli et al., 2016; Ghanavati et al., 2020). Although most studies demonstrate the protective role of *Lactobacilli* products, lactic acid has conflicting results. Lactate and butyrate inhibit HDACs, thereby promoting histone acetylation by histone acetylases. Acetylated histones induce chromatin relaxation, resulting in an increased DNA repair rate (Wagner et al., 2015; Wagner et al., 2017), and recruitment of DNA-dependent protein kinase catalytic subunits (DNA-PKcs) to the nucleus. Cervical epithelial cells bear the lactate hydroxycarboxylic acid receptor 1 (HCA1/GPR81). Lactate stimulates surface HCA1 and induces cAMP signalling and the cAMP/EPAC/PKA-dependent shuttling of DNA-PKcs. DNA-PKcs sense foreign or damaged self-DNA and are essential components of the non-homologous end-joining pathway which protects cells from lentiviral (HPV and HIV-1) transduction, thus restricting viral oncogenic and/or cytopathic potential (Wagner et al., 2021). In addition, core fucosylation levels are significantly reduced in the serum, exfoliated cervical cells, and tumour tissues from patients with cervical cancer. *L. iners* metabolites and lactate activate the Wnt pathway by means of the lactic acid-HCA1 complex, increase the level of core fucosylation, thereby inhibiting the proliferation and migration of cervical cancer cells (Fan et al., 2021a). In contrast, lactic acid (a major metabolite in glycolytic tumour cells) can be imported to fuel mitochondrial respiration (Warburg, 1956). Lactate induces the expression of TGF-β2 in gastric and lung cancers which upregulates the expression and activation of MMP-2, thereby promoting tumour cell metastasis. It also promotes angiogenesis by upregulating HIF-1α and VEGF (Kolev et al., 2008). In addition, the immunomodulatory effect of lactate on T cells, macrophages, and CTL may lead to tumour immune escape (Colegio et al., 2014; Brand et al., 2016). Detection of high lactate levels among human cervical cancers predicted tumour metastasis, recurrence and restricted of patient survival (Walenta et al., 2000). Li et al. demonstrated that lactate induces migration and invasion of SiHa cells *via* the miR-774/ARHGAP5 axis, although the effect is diminished due to partial inhibition of E6 and E7 expression (Li et al., 2019). However, the exact roles of lactic acid in cervical cancer with respect to HPV protein expression, proliferation, and migration of cancer cells remain unclear.

HIV infection

Most studies show that BV and low *Lactobacilli* CVM are associated with an increased risk of HIV acquisition (Atashili et al., 2008), transmission (Cohen et al., 2012), and a risk factor for intrapartum HIV trasmission(Atashili et al., 2008), and adverse pregnancy outcome(Short et al., 2020; Mwenda et al., 2021). A nested case-control study including five cohorts shows concentration-dependent associations between *Eggerthella species type 1, Gemella asaccharolytica*, *Leptotrichia/Sneathia*, *Megasphaera* spp., and *Mycoplasma hominis* with increased odds of HIV acquisition (Eastment and McClelland, 2018). Other studies also demonstrate that *Prevotella melaninogenica, V. montpellierensis, Mycoplasma, P. bivia*, *Sneathia sanguinegens* (Gosmann et al., 2017), and L.iners (Taku et al., 2022) are associated with HIV acquisition. HIV with unsuppressed viral loads are more likely to have higher abundance of *Megasphaera genomosp.1*, *A. vaginae*, and *Clostridiales* sp. compared to healthy and recurrent BV patients (Elwood et al., 2020). Relative studies are summarized in Supplementary Table 2.

The impact of the cervix immune response induced by the BV-associated microbiome on HIV acquisition is complicated and conflicting. On the one hand, the increase in BV-associated microbiota and inflammatory cytokines indicates a disruption of the epithelial barrier associated with HIV genital tract shedding and the recruitment of HIV target cells. Abnormally high levels of several cytokines such as IL-1β, IL-6, MIP-1α, and TNF-α are associated with HIV disease progression (Herold et al., 2013) and contribute to HIV persistence during antiretroviral treatment (ART) by promoting residual levels of viral production (Vandergeeten et al., 2012). BV-associated flora are associated with endo- (Gosmann et al., 2017) and ectocervix (Thurman et al., 2015) $CD4^+CCR5^+$T cells compared to healthy flora. Elevated inflammatory cytokine levels are associated with an increased frequency of cervical $CD4^+$T cell (Arnold et al., 2016; Gosmann et al., 2017). Activated $CD4^+$T cells and GD T cells are affected by vaginal flora and are associated with HIV infection. GD1 T cells are protective in the genital tract and GD2 T cells are targets for HIV entry. Cervical GD1 T cells and GD2 T cells are lower and higher in women with BV than in those with normal vaginal flora, respectively which predisposes women with HIV acquisition (Alcaide et al., 2016). On the other hand, mucosal IgG and IgM elevated with genital inflammation, IgM neutralises and reduces HIV infections and directly binds to $CD4^+$T cells and chemokine receptor

CCR5, hindering HIV entry (Sobia et al., 2021). Some bacteria, such as *L. gasseri*, *L. crispatus*, *V. montpellierensis*, *P. amnii*, and *S. amnii* are positively correlated with the frequency of mucosal-activated natural killer (NK) cells that coordinate the early control of HIV infection (Munusamy et al., 2021). The exact effect of CVM on HIV infection *via* local immune responses remains further explored.

CVM also plays a role in the pharmacokinetics of pre-exposure prophylaxis (PrEP) drugs. However, previous studies reported inconsistent results on this. Some clinical trials using topical 1% tenofovir (TFV) vaginal gel in the CAPRISA 004 cohort and TFV vaginal film in the FAME 04 cohort found that women with low *Lactobacilli* flora have reduced mucosal concentrations of TFV, which potentially reduces the efficacy of PrEP drugs (Klatt et al., 2017). BV cervicovaginal larvae and *G. vaginalis* reduce the uptake of PrEP drugs by Jurkat cells, and increase TFV degradation and HIV infection compared to normal cervicovaginal larvae and *L. crispatus* (Cheu et al., 2020). However, another study examined the effect of CVM on TFV vaginal ring which can continuously deliver for approximately 14 days, and showed a positively correlates between CST IVA/B and high level of TFV during the first two months of use (Thurman et al., 2022). Nevertheless, individuals with a high abundance of *Prevotella* spp. or *G. vaginalis* show reduced TFV/TFV-diphosphate concentrations in the vaginal fluid and tissues in the third month. In addition, pH drives TFV release since it affects TFV transport into human cells (Johnson et al., 2012; Taneva et al., 2018). The authors speculate that a higher pH *in vivo* increases aqueous solubility, and more TFV will dissolve from the formulated paste inside the vaginal ring, which may explain the positive correlation between TFV and CST IVA/B in the first two months.

Protonated L-lactic acid is a major anti-HIV metabolite of Lactobacilli (Kyongo et al., 2015; Ñahui et al., 2017; Tyssen et al., 2018). 0.3% w/w L-lactic acid shows a 17 times higher anti-HIV activity than that of D-lactic acid. Moreover, a physiological concentration of L-lactic acid can inactivate HIV in the presence of 50% cervix vaginal secretions and 0.75% semen. The anti-HIV activity of L-lactic acid is pH dependent, and can be abrogated under neutral pH (Aldunate et al., 2013). Healthy cervicovaginal mucus inhibits HIV activity and transmission. HIV spreads in the form of free viral particles and cellular contact. Although L-lactic acid has a stronger ability to inhibit HIV activity than D-lactic acid, the HIV-1 virion can be greatly trapped in CVM with high D-lactic acid concentration and an *L. crispatus*-dominant microbiota (Nunn et al., 2015). HIV virions have increased mobility in *L. iners* dominant mucus and BV mucus which is related to the levels of D-lactic acid and total lactic acid. The trapping of HIV-1 viruses in the cervicovaginal mucus is mainly based on adhesive interactions. The addition of lactic acid to the cervicovaginal mucus with reduced adhesive properties did not restore HIV adhesion (Nunn et al., 2015). Therefore, the reduction in mucoadhesive interactions might be due to an increased amount of mucin-degrading enzymes that Thus, the reduction in adhesive interactions may be due to elevated levels of mucin-degrading enzymes which disturb HIV-mucin interactions.

The release of extracellular vesicles (EVs) is another approach which protects HIV infection mediated by Lactobacilli (Ñahui et al., 2019). EVs constitute a lipid bilayer membrane carrying numerous bioactive molecules and are considered to be important mediators of cell–cell communication (Costantini et al., 2022; Nagakubo et al., 2019). *Lactobacillus*-derived EVs (*L. crispatus* BC3 and *L. gasseri* BC12) bind to the HIV-1 Env protein, leading to a reduction of HIV-1 entry and binding to target cells. Nevertheless, EVs released by other gram-positive species (Staphylococcus aureus, G. vaginalis, Enterococcus faecium, and Enterococcus faecalisi) also protect human cervicovaginal tissues ex vivo and isolated cells from HIV-1 infection by blocking HIV-1-cell receptor interactions which are related to the steric hindrance of gp120 or gp120 modification (Costantini et al., 2022).

Confounders affect microbiome sequencing

Several confounders may account for the discrepancies across studies among the clinical sample sequencing researches. First, the difference in the baseline characters of subjects, such as ethnicity and geographical region, age, menstrual state, previous hormone and antibiotic treatment history can influence the composition of the reproductive tract microbiome. One example is that some studies about microbiota sequencing on CT infection show inconsistence on Aerococcus christensenii and diversity (Balle et al., 2018; Di Pietro et al., 2018; Cheong et al., 2019). Confounders such as different region, ethnicity and age may account for these bias. Second, sampling site and methodology may also have influence on microbiome sequencing as well as cytokines detection. As discussed before, there are difference in the microbiome composition of vagina wall, posterior fornix and endocervix, as well as sampling device (Kim et al., 2009; Virtanen et al., 2017; Kero et al., 2023). Cytokine and protein concentration were also different collected by endocervical swabs, lavage samples, and vaginal swabs (Dezzutti et al., 2011). Third, sequencing method, laboratory and data processing differ across studies, such as DNA extraction, sequencing target, platform, and annotation database may also impact the sequencing, metabolome and proteome result (Theodoridis et al., 2012; Schnaars et al., 2018; Mattei et al., 2019; Li and Sui, 2021; Sirichoat et al., 2021). For instance, the richness and diversity of cervical microbiota increased in HR-HPV positive women compared with healthy control by 16S rRNA sequencing, however, the difference were not obvious in metagenomic sequencing (Fang et al., 2022). Despite the discrepancies in the result of some specific bacteria in different sequencing studies due to these confounders, presence or increased relative abundances of Lactobacillus spp. are generally associated with decreased risk of female reproductive STI; BV (CST IV vaginal microbiota), and particular BVAB have been found to be associated with increased risk.

Conclusion and outlook

Recent accumulating studies have shown complex interactions between CVM, metabolites, and host cells in the cervical ecosystem. Here, we focused on the crosstalk between CVM and cervical epithelial-, immune-, and mucus barriers.

Generally, *Lactobacillus*-dominant microbiota exhibit immunomodulatory effects on cervical epithelial and immune cells, maintain cervical epithelial integrity, and protect cervical CT, HPV, and HIV infection with respect to infection, treatment, and clearance.

CVM dysbiosis induces an inflammatory response in cervical epithelial and immune cells in microbe-specific modes and directly induces epithelial barrier disruption by inducing oxidative stress, miRNA alteration, and promoting cell cycle arrest, apoptosis, and necrosis. Furthermore, an indirect response may occur through the secretion of harmful metabolites and causes immune disorders. Dysregulation of cervical cytokine and immune cell profiles induced by CVM dysbiosis is also associated with STI infection and relative disease development. *Lactobacillus* binds mucin, while BVAB stimulates mucin expression and degrades these highly glycosylated proteins which decrease the ability to trap and remove pathogens. BVABs also degrade collagen, gelatin, and casein, and alter cervical biomechanical properties which cause rapid cervical remodelling and harm human pregnancy.

Lactic acid is the most well-studied and potent normal CVM metabolite in maintaining cervicovaginal barrier homeostasis, and inhibiting CT infection and HIV activity; however, its exact role in HPV infection and cervical cancer remains unclear. The specific mechanisms involved in the *Lactobacillus*, culture supernatant and lactic acid in anti-STI infection mainly include 1) reduces polar lipids and α5β1 integrin subunit exposure in the epithelial plasma membrane, inhibit EB binding and invasion in epithelial cell; 2) inhibit HDACs so that inhibit host cell proliferation, and induce chromatin relaxation, increase DNA repair rate; 3) recruit DNA-PKcs to the nucleus, which can sense foreign or damaged self-DNA, protects cells from lentiviral (HPV and HIV-1) transduction; 4) reduce the expression of autophagy genes and HPV E6 and E7 to inhibit cervical cancer cells activity. Contradictory mechanisms mainly include 1) can be used as fuel mitochondrial respiration in glycolytic tumour cells; 2) promote MMP-2, HIF-1α, VEGF, miR-774/ARHGAP5 axis which promote metastasis, angiogenesis, migration and invasion; 3) immunomodulatory effect on T cells, macrophages, and CTL may lead to tumour immune escape. The association between BVAB, culture supernatant and SCFAs with STI infection usually related with the cytokines change and immune cells recruitment induced by the inflammatory response. The specific mechanisms in the influence on cervical STI disease are less documented.

Going forward, although some studies have observed the interactions between CVM, their metabolites, with host microenvironment using omics technology, the mechanistic understanding towards how these microbiota and their metabolites influence STI infections are lack. For instance, the bioactive metabolites which can interact with bacteria, STI pathogens and host epithelial and immune cell and are less identified, as well as the possible passways. A better understanding can provide information in the developing and optimizing of diagnostics, treatment strategies and drugs, probiotics, postbiotics and vaginal flora transplantation. The innumerable hypotheses generated from these omics studies should be verified by *in vitro* and *in vivo* studies. A major gap towards these issue is a lack of suitable *in vitro* and animal experimental models (Bjornson-Hooper et al., 2022; Mahajan et al., 2022). The microbiome, cervicovaginal epithelium and immune reaction differ between human, mice and non-human primates. Exploring suitable models simulate female reproductive tract environment is essential to better understand the intimate interaction between CVM, their metabolites and cervical host cell. Advanced tools including metagenomics, metatranscriptomics, and metabolomics will provide insights into the characteristics of functional microbiomes and metabolites, determine the potential mechanisms used to connect with host cells, and further form interaction networks in health and disease. Future studies involving molecular detection technology in human samples, cells, and animal biological studies will aid in the discovery of novel diagnostic and therapeutic targets for female reproductive diseases.

Author contributions

MD, CH, and FX conceived the study question, and all authors were involved in the study design. MD,YD created the first draft of the manuscript. JB, HuaL, XM, BL, CW, HuiL, and AF made substantial contributions to drafting the article and revising it critically. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1124591/full#supplementary-material

References

Aflatoonian, R., and Fazeli, A. (2008). Toll-like receptors in female reproductive tract and their menstrual cycle dependent expression. *J. Reprod. Immunol.* 77 (1), 7–13. doi: 10.1016/j.jri.2007.03.014

Ahire, J. J., Sahoo, S., Kashikar, M. S., Heerekar, A., Lakshmi, S. G., and Madempudi, R. S. (2021). In vitro assessment of lactobacillus crispatus UBLCp01, lactobacillus gasseri UBLG36, and lactobacillus johnsonii UBLJ01 as a potential vaginal probiotic candidate. *Probiotics Antimicrob. Proteins*. doi: 10.1007/s12602-021-09838-9

Aiyar, A., Quayle, A. J., Buckner, L. R., Sherchand, S. P., Chang, T. L., Zea, A. H., et al. (2014). Influence of the tryptophan-indole-IFNγ axis on human genital chlamydia trachomatis infection: role of vaginal co-infections. *Front. Cell Infect. Microbiol.* 4. doi: 10.3389/fcimb.2014.00072

Alcaide, M. L., Strbo, N., Romero, L., Jones, D. L., Rodriguez, V. J., Arheart, K., et al. (2016). Bacterial vaginosis is associated with loss of gamma delta T cells in the female reproductive tract in women in the Miami women interagency HIV study (WIHS): A cross sectional study. *PloS One* 11 (4), e0153045. doi: 10.1371/journal.pone.0153045

Aldunate, M., Tyssen, D., Johnson, A., Zakir, T., Sonza, S., Moench, T., et al. (2013). Vaginal concentrations of lactic acid potently inactivate HIV. *J. Antimicrob. Chemother.* 68 (9), 2015–2025. doi: 10.1093/jac/dkt156

Amabebe, E., and Anumba, D. O. C. (2018). The vaginal microenvironment: The physiologic role of lactobacilli. *Front. Med.* 5. doi: 10.3389/fmed.2018.00181

Anahtar, M. N., Byrne, E. H., Doherty, K. E., Bowman, B. A., Yamamoto, H. S., Soumillon, M., et al. (2015). Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* 42 (5), 965–976. doi: 10.1016/j.immuni.2015.04.019

Andersch-Björkman, Y., Thomsson, K. A., Holmén, L. J. M., Ekerhovd, E., and Hansson, G. C. (2007). Large Scale identification of proteins, mucins, and their O-glycosylation in the endocervical mucus during the menstrual cycle. *Mol. Cell Proteomics* 6 (4), 708–716. doi: 10.1074/mcp.M600439-MCP200

Anton, L., DeVine, A., Sierra, L.-J., Brown, A. G., and Elovitz, M. A. (2017). miR-143 and miR-145 disrupt the cervical epithelial barrier through dysregulation of cell adhesion, apoptosis and proliferation. *Sci. Rep.* 7 (1), 3020. doi: 10.1038/s41598-017-03217-7

Anton, L., Ferguson, B., Friedman, E. S., Gerson Kristin, D., Brown Amy, G., and Elovitz, M. A. (2022). Gardnerella vaginalis alters cervicovaginal epithelial cell function through microbe-specific immune responses. *Microbiome* 10 (1), 119. doi: 10.1186/s40168-022-01317-9

Anton, L., Sierra, L.-J., DeVine, A., Barila, G., Heiser, L., Brown Amy, G., et al. (2018). Lactobacillus crispatusCommon cervicovaginal microbial supernatants alter cervical epithelial function: Mechanisms by which contributes to cervical health. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.02181

Arnold, K. B., Burgener, A., Birse, K., Romas, L., Dunphy, L. J., Shahabi, K., et al. (2016). Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells. *Mucosal Immunol.* 9 (1), 194–205. doi: 10.1038/mi.2015.51

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., Deroos, P., et al. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504 (7480), 451–455. doi: 10.1038/nature12726

Atashili, J., Poole, C., Ndumbe, P. M., Adimora Adaora, A., and Smith, J. S. (2008). Bacterial vaginosis and HIV acquisition: A meta-analysis of published studies. *AIDS* 22 (12), 1493–1501. doi: 10.1097/QAD.0b013e3283021a37

Balle, C., Konstantinus, I. N., Jaumdally, S. Z., Havyarimana, E., Lennard, K., Esra, R., et al. (2020). Hormonal contraception alters vaginal microbiota and cytokines in south African adolescents in a randomized trial. *Nat. Commun.* 11 (1), 5578. doi: 10.1038/s41467-020-19382-9

Balle, C., Lennard, K., Dabee, S., Barnabas, S. L., Jaumdally, S. Z., Gasper, M. A., et al. (2018). Endocervical and vaginal microbiota in south African adolescents with asymptomatic chlamydia trachomatis infection. *Sci. Rep.* 8 (1), 11109. doi: 10.1038/s41598-018-29320-x

Beatty, W. L., Belanger, T. A., Desai, A. A., Morrison, R. P., and Byrne, G. I. (1994). Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. *Infect. Immun.* 62 (9), 3705–3711. doi: 10.1128/iai.62.9.3705-3711.1994

Benjelloun, F., Quillay, H., Cannou, C., Marlin, R., Madec, Y., Fernandez, H., et al. (2020). Activation of toll-like receptors differentially modulates inflammation in the human reproductive tract: Preliminary findings. *Front. Immunol.* 11. doi: 10.3389/fimmu.2020.01655

Bjornson-Hooper, Z. B., Fragiadakis, G. K., Spitzer, M. H., Chen, H., Madhireddy, D., Hu, K., et al. (2022). A comprehensive atlas of immunological differences between humans, mice, and non-human primates. *Front. Immunol.* 13. doi: 10.3389/fimmu.2022.867015

Blaskewicz, C. D., Pudney, J., and Anderson, D. J. (2011). Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia. *Biol. Reprod.* 85 (1), 97–104. doi: 10.1095/biolreprod.110.090423

Boily-Larouche, Geneviève., Lajoie, J., Dufault, B., Omollo, K., Cheruiyot, J., Njoki, J., et al. (2019). Characterization of the genital mucosa immune profile to distinguish phases of the menstrual cycle: Implications for HIV susceptibility. *J. Infect. Dis.* 219 (6), 856–866. doi: 10.1093/infdis/jiy585

Borgdorff, H., Gautam, R., Armstrong, S. D., Xia, D., Ndayisaba, G. F., van Teijlingen, N. H., et al. (2016). Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier. *Mucosal Immunol.* 9 (3), 621–633. doi: 10.1038/mi.2015.86

Borgogna, J. C., Shardell, M. D., Santori, E. K., Nelson, T. M., Rath, J. M., Glover, E. D., et al. (2020). The vaginal metabolome and microbiota of cervical HPV-positive and HPV-negative women: A cross-sectional analysis. *BJOG* 127 (2), 182–192. doi: 10.1111/1471-0528.15981

Boukari, Hacène., Brichacek, B., Stratton, P., Mahoney, S. F., Lifson, J. D., Margolis, L., et al. (2009). Movements of HIV-virions in human cervical mucus. *Biomacromolecules* 10 (9), 2482–2488. doi: 10.1021/bm900344q

Bradley, F., Birse, K., Hasselrot, K., Noël-Romas, L., Introini, A., Wefer, H., et al. (2018). The vaginal microbiome amplifies sex hormone-associated cyclic changes in cervicovaginal inflammation and epithelial barrier disruption. *Am. J. Reprod. Immunol.* 80 (1), e12863. doi: 10.1111/aji.12863

Bradshaw, C. S., Tabrizi, S. N., Fairley, C. K., Morton, A. N., Rudland, E., and Garland, S. M. (2006). The association of atopobium vaginae and gardnerella vaginalis with bacterial vaginosis and recurrence after oral metronidazole therapy. *J. Infect. Dis.* 194 (6), 828–836. doi: 10.1086/506621

Brand, A., Singer, K., Koehl, G. E., Kolitzus, M., Schoenhammer, G., Thiel, A., et al. (2016). LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. *Cell Metab.* 24 (5), 657–671. doi: 10.1016/j.cmet.2016.08.011

Brotman, R. M., Shardell, M. D., Gajer, P., Tracy, J.K., Zenilman, J. M., Ravel, J., et al. (2014). Interplay between the temporal dynamics of the vaginal microbiota and human papillomavirus detection. *J. Infect. Dis.* 210 (11), 1723–1733. doi: 10.1093/infdis/jiu330

Brunham, R. C., and Rekart, M. L. (2009). Considerations on chlamydia trachomatis disease expression. *FEMS Immunol. Med. Microbiol.* 55 (2), 162–166. doi: 10.1111/j.1574-695X.2008.00509.x

Chen, H., Min, S., Wang, Li., Zhao, L., Luo, F., Lei, W., et al. (2022). Lactobacillus modulates chlamydia infectivity and genital tract pathology in vitro and *in vivo*. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.877223

Chen, Y., Qiu, X., Wang, W., Li, D., Wu, A., Hong, Z., et al. (2020). Human papillomavirus infection and cervical intraepithelial neoplasia progression are associated with increased vaginal microbiome diversity in a Chinese cohort. *BMC Infect. Dis.* 20 (1), 629. doi: 10.1186/s12879-020-05324-9

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., et al. (2017). The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat. Commun.* 8 (1), 875. doi: 10.1038/s41467-017-00901-0

Chen, H., Wang, Li., Zhao, L., Luo, L., Min, S., Wen, Y., et al. (2021). Chlamydia trachomatisAlterations of vaginal microbiota in women with infertility and infection. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.698840

Cheong, H. C., Yap, P. S. X., Chong, C. W., Cheok, Y. Y., Lee, C. Y. Q., Tan, G. M. Y., et al. (2019). Diversity of endocervical microbiota associated with genital chlamydia trachomatis infection and infertility among women visiting obstetrics and gynecology clinics in Malaysia. *PloS One* 14 (11), e0224658. doi: 10.1371/journal.pone.0224658

Cheu, R. K., Gustin, A. T., Lee, C., Schifanella, L., Miller, C. J., Ha, A., et al. (2020). Impact of vaginal microbiome communities on HIV antiretroviral-based pre-exposure prophylaxis (PrEP) drug metabolism. *PloS Pathog.* 16 (12), e1009024. doi: 10.1371/journal.ppat.1009024

Chiu, S.-F., Huang, P.-J., Cheng, W.-H., Huang, C.-Y., Chu, L. J., Lee, C.-C., et al. (2021). Chlamydia trachomatis vaginal microbiota of the sexually transmitted infections caused by and in women with vaginitis in Taiwan. *Microorganisms* 9 (9), 1864. doi: 10.3390/microorganisms9091864

Cohen, C. R., Lingappa, J. R., Baeten, J. M., Ngayo, M. O., Spiegel, C. A., Hong, T., et al. (2012). Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: A prospective cohort analysis among African couples. *PloS Med.* 9 (6), e1001251. doi: 10.1371/journal.pmed.1001251

Colegio, O. R., Chu, N.-Q., Szabo, A. L., Chu, T., Rhebergen, A. M., Jairam, V., et al. (2014). Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* 513 (7519), 559–563. doi: 10.1038/nature13490

Costantini, P. E., Vanpouille, C., Firrincieli, A., Cappelletti, M., Margolis, L., and Ñahui Palomino, R. A. (2022). Extracellular vesicles generated by gram-positive bacteria protect human tissues *ex vivo* from HIV-1 infection. *Front. Cell. infection Microbiol.* 11. doi: 10.3389/fcimb.2021.822882

Cruciani, F., Wasinger, V., Turroni, S., Calanni, F., Donders, G., Brigidi, P., et al. (2013). Proteome profiles of vaginal fluids from women affected by bacterial vaginosis and healthy controls: outcomes of rifaximin treatment. *J. Antimicrob. Chemother.* 68 (11), 2648–2659. doi: 10.1093/jac/dkt244

Delgado-Diaz, D. J., Jesaveluk, B., Hayward, J. A., Tyssen, D., Alisoltani, A., Potgieter, M., et al. (2022). Lactic acid from vaginal microbiota enhances cervicovaginal epithelial barrier integrity by promoting tight junction protein expression. *Microbiome* 10 (1), 141. doi: 10.1186/s40168-022-01337-5

Delgado-Diaz, D. J., Tyssen, D., Hayward, J. A., Gugasyan, R., Hearps, A. C., and Tachedjian, G. (2019). Distinct immune responses elicited from cervicovaginal epithelial cells by lactic acid and short chain fatty acids associated with optimal and non-optimal vaginal microbiota. *Front. Cell Infect. Microbiol.* 9 (undefined). doi: 10.3389/fcimb.2019.00446

Deruaz, M., and Luster, A. D. (2015). Chemokine-mediated immune responses in the female genital tract mucosa. *Immunol. Cell Biol.* 93 (4), 347–354. doi: 10.1038/icb.2015.20

De Tomasi, J. B., Opata, M. M., and Chishimba, N. M. (2019). Immunity in the cervix: Interphase between immune and cervical epithelial cells. *J. Immunol. Res.* 2019 (undefined), 7693183. doi: 10.1155/2019/7693183

Dezzutti, C. S., Hendrix, C. W., Marrazzo, J. M., Pan, Z., Wang, L., Louissaint, N., et al. (2011). Performance of swabs, lavage, and diluents to quantify biomarkers of female genital tract soluble mucosal mediators. *PloS One* 6 (8), e23136. doi: 10.1371/journal.pone.0023136

Di Paola, M., Seravalli, V., Paccosi, S., Linari, C., Parenti, A., De Filippo, C., et al. (2020). Identification of vaginal microbial communities associated with extreme cervical shortening in pregnant women. *J. Clin. Med.* 9 (11), 3621. doi: 10.3390/jcm9113621

Di Pietro, M., Filardo, S., Porpora, M. G., Recine, N., Latino, M. A., and Sessa, R. (2018). HPV/Chlamydia trachomatis co-infection: Metagenomic analysis of cervical microbiota in asymptomatic women. *New Microbiol.* 41 (1), 34–41.

Dong, B., Huang, Y., Cai, H., Chen, Y., Li, Y., Zou, H., et al. (2022). Prevotella as the hub of the cervicovaginal microbiota affects the occurrence of persistent human papillomavirus infection and cervical lesions in women of reproductive age via host NF-κB/C-myc. *J. Med. Virol.* 94 (11), 5519–5534. doi: 10.1002/jmv.28001

Dude, C. M., Saylany, A., Brown, A., Elovitz, M., and Anton, L. (2020). Microbial supernatants from mobiluncus mulieris, a bacteria strongly associated with spontaneous preterm birth, disrupts the cervical epithelial barrier through inflammatory and miRNA mediated mechanisms. *Anaerobe* 61, 102127. doi: 10.1016/j.anaerobe.2019.102127

Eade, C. R., Diaz, C., Wood, M. P., Anastos, K., Patterson, B. K., Gupta, P., et al. (2012). Identification and characterization of bacterial vaginosis-associated pathogens using a comprehensive cervical-vaginal epithelial coculture assay. *PloS One* 7 (11), e50106. doi: 10.1371/journal.pone.0050106

Eastment, M. C., and McClelland, R. S. (2018). *Vaginal microbiota and susceptibility to HIV* (London, England: AIDS) 32, 687–698. doi: 10.1097/QAD.0000000000001768

Edwards, V. L., Smith, S. B., McComb, E. J., Tamarelle, J., Ma, B., Humphrys, M. S., et al. (2019). The cervicovaginal microbiota-host interaction modulates chlamydia trachomatis infection. *mBio* 10 (4), e01548–19. doi: 10.1128/mBio.01548-19

Elwood, C., Albert, A., McClymont, E., Wagner, E., Mahal, D., Devakandan, K., et al. (2020). Different and diverse anaerobic microbiota were seen in women living with HIV with unsuppressed HIV viral load and in women with recurrent bacterial vaginosis: A cohort study. *BJOG* 127 (2), 250–259. doi: 10.1111/1471-0528.15930

Fan, Q., Wu, Y., Li, M., An, F., Yao, L., Wang, M., et al. (2021). Lactobacillus spp. create a protective micro-ecological environment through regulating the core fucosylation of vaginal epithelial cells against cervical cancer. *Cell Death Dis.* 12 (12), 1094. doi: 10.1038/s41419-021-04388-y

Fang, B., Li, Q., Wan, Z., Ouyang, Z., and Zhang, Q. (2022). Exploring the association between cervical microbiota and HR-HPV infection based on 16S rRNA gene and metagenomic sequencing. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.922554

Farr Zuend, N. C., Tobin Nicole, H., Vera, T., Kotyrba, L, Noël-Romas, L., Birse, K., et al. (2020). Pregnancy associates with alterations to the host and microbial proteome in vaginal mucosa. *Am. J. Reprod. Immunol.* 83 (6), e13235. doi: 10.1111/aji.13235

Ferreira, C. S. T., da Silva, MárciaGuimarães., de Pontes, LetíciaG., Dos Santos, L. D., and Marconi, C. (2018). Protein content of cervicovaginal fluid is altered during bacterial vaginosis. *J. Low Genit Tract Dis.* 22 (2), 147–151. doi: 10.1097/LGT.0000000000000367

Ferreira, C. S. T., Donders, G. G., Parada, C., Tristão, A. D. R., Fernandes, T., Da Silva, M. G., et al. (2017). Treatment failure of bacterial vaginosis is not associated with higher loads of atopobium vaginae and gardnerella vaginalis. *J. Med. Microbiol.* 66 (8), 1217–1224. doi: 10.1099/jmm.0.000561

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., et al. (2019). The vaginal microbiome and preterm birth. *Nat. Med.* 25 (6), 1012–1021. doi: 10.1038/s41591-019-0450-2

Filardo, S., Di Pietro, M., Porpora, M. G., Recine, N., Farcomeni, A., Latino, M. A., et al. (2017). Diversity of cervical microbiota in asymptomatic chlamydia trachomatis genital infection: A pilot study. *Front. Cell. infection Microbiol.* 7. doi: 10.3389/fcimb.2017.00321

Filardo, S., Di Pietro, M., Tranquilli, G., Latino, M. A., Recine, N., Porpora, M. G., et al. (2019). Selected immunological mediators and cervical microbial signatures in women with chlamydia trachomatis infection. *mSystems* 4 (4), e00094–19. doi: 10.1128/mSystems.00094-19

France, M. T., Ma, B., Gajer, P., Brown, S., Humphrys, M. S., Holm, J. B., et al. (2020). VALENCIA: A nearest centroid classification method for vaginal microbial communities based on composition. *Microbiome* 8 (1), 166. doi: 10.1186/s40168-020-00934-6

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schütte, U. M., Zhong, X., et al. (2012). Temporal dynamics of the human vaginal microbiota. *Sci. Trans. Med.* 4 (132), 132ra52. doi: 10.1126/scitranslmed.3003605

Gardner, J. K., Łaniewski, Paweł., Knight, A., Haddad, L. B., Swaims-Kohlmeier, A., and Herbst-Kralovetz, M. M. (2020). Interleukin-36γ is elevated in cervicovaginal epithelial cells in women with bacterial vaginosis and in vitro after infection with microbes associated with bacterial vaginosis. *J. Infect. Dis.* 221 (6), 983–988. doi: 10.1093/infdis/jiz514

Gebhardt, T., Mueller, S. N., Heath, W. R., and Carbone, F. R. (2013). Peripheral tissue surveillance and residency by memory T cells. *Trends Immunol.* 34 (1), 27–32. doi: 10.1016/j.it.2012.08.008

Gerson, K. D., Anton, L., Ferguson, B., Ravel, J., Burris, H. H., and Elovitz, M. A. (2022). Gardnerella vaginalis induces matrix metalloproteinases in the cervicovaginal epithelium through TLR-2 activation. *J. Reprod. Immunol.* 152, 103648. doi: 10.1016/j.jri.2022.103648

Ghanavati, R., Asadollahi, P., Shapourabadi, M. B., Razavi, S., Talebi, M., and Rohani, M. (2020). Inhibitory effects of lactobacilli cocktail on HT-29 colon carcinoma cells growth and modulation of the notch and wnt/β-catenin signaling pathways. *Microb. Pathog.* 139, 103829. doi: 10.1016/j.micpath.2019.103829

Ghosh, M., Rodriguez-Garcia, M., and Wira, C. R. (2014). The immune system in menopause: pros and cons of hormone therapy. *J. Steroid Biochem. Mol. Biol.* 142, 171–175. doi: 10.1016/j.jsbmb.2013.09.003

Ghosh, M., Shen, Z., Fahey, J. V., Crist, S. G., Patel, M., Smith, J. M., et al. (2013). Pathogen recognition in the human female reproductive tract: Expression of intracellular cytosolic sensors NOD1, NOD2, RIG-1, and MDA5 and response to HIV-1 and neisseria gonorrhea. *Am. J. Reprod. Immunol.* 69 (1), 41–51. doi: 10.1111/aji.12019

Gipson, I. K., Moccia, R., Spurr-Michaud, S., Argüeso, P., Gargiulo, A. R., Hill, J. A., et al. (2001). The amount of MUC5B mucin in cervical mucus peaks at midcycle. *J. Clin. Endocrinol. Metab.* 86 (2), 594–600. doi: 10.1210/jcem.86.2.7174

Gipson, I. K., Spurr-Michaud, S., Moccia, R., Zhan, Q., Toribara, N., Ho, S. B., et al. (1999). MUC4 and MUC5B transcripts are the prevalent mucin messenger ribonucleic acids of the human endocervix. *Biol. Reprod.* 60 (1), 58–64. doi: 10.1095/biolreprod60.1.58

Gliniewicz, K., Schneider, G. M., Ridenhour, B. J., Williams, C. J., Song, Y., Farage, M. A., et al. (2019). Comparison of the vaginal microbiomes of premenopausal and postmenopausal women. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.00193

Gosmann, C., Anahtar, M. N., Handley, S. A., Farcasanu, M., Abu-Ali, G., Bowman, B. A., et al. (2017). Lactobacillus-deficient cervicovaginal bacterial communities are associated with increased HIV acquisition in young south African women. *Immunity* 46 (1), 29–37. doi: 10.1016/j.immuni.2016.12.013

Grant, K. S., and Wira, C. R. (2003). Effect of mouse uterine stromal cells on epithelial cell transepithelial resistance (TER) and TNFalpha and TGFbeta release in culture. *Biol. Reprod.* 69 (3), 1091–1098. doi: 10.1095/biolreprod.103.015495

Gustin, A. T., Thurman Andrea, R., Chandra, N., Schifanella, L., Alcaide, M., Fichorova, R., et al. (2022). Recurrent bacterial vaginosis following metronidazole treatment is associated with microbiota richness at diagnosis. *Am J Obstet Gynecol.* 226 (2), 225.e1–225.e15. doi: 10.1016/j.ajog.2021.09.018

Hall, O. J., and Klein, S. L. (2017). Progesterone-based compounds affect immune responses and susceptibility to infections at diverse mucosal sites. *Mucosal Immunol.* 10 (5), 1097–1107. doi: 10.1038/mi.2017.35

Han, L., Park, D., Reddy, A., Wilmarth, P. A., and Jensen, J. T. (2021). Comparing endocervical mucus proteome of humans and rhesus macaques. *Proteomics Clin. Appl.* 15 (4), e2100023. doi: 10.1002/prca.202100023

Han, L., Taub, R., and Jensen, J. T. (2017). Cervical mucus and contraception: what we know and what we don't. *Contraception* 96 (5), 310–321. doi: 10.1016/j.contraception.2017.07.168

Hansson, G. C. (2020). Mucins and the Microbiome. *Annu Rev Biochem*, 89 (undefined), 769–793. doi: 10.1146/annurev-biochem-011520-105053

Hearps, A. C., Tyssen, D., Srbinovski, D., Bayigga, L., Diaz, D. J. D., Aldunate, M., et al. (2017). Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition. *Mucosal Immunol.* 10 (6), 1480–1490. doi: 10.1038/mi.2017.27

Herold, B. C., Keller, M. J., Shi, Q., Hoover, D. R., Carpenter, C. A., Huber, A., et al. (2013). Plasma and mucosal HIV viral loads are associated with genital tract inflammation in HIV-infected women. *J. Acquir. Immune Defic. Syndr.* 63 (4), 485–493. doi: 10.1097/QAI.0b013e3182961cfc

Hickey, D. K., Patel, M. V., Fahey, J. V., and Wira, C. R. (2011). Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J. Reprod. Immunol.* 88 (2), 185–194. doi: 10.1016/j.jri.2011.01.005

Hladik, F., Sakchalathorn, P., Ballweber, L., Lentz, G., Fialkow, M., Eschenbach, D., et al. (2007). Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. *Immunity* 26 (2), 257–270. doi: 10.1016/j.immuni.2007.01.007

Hunter, P. J., Sheikh, S., David, A. L., Peebles, D. M., and Klein, N. (2016). Cervical leukocytes and spontaneous preterm birth. *J. Reprod. Immunol.* 113, 42–49. doi: 10.1016/j.jri.2015.11.002

Iijima, N., and Iwasaki, A. (2014). T Cell memory. a local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* 346 (6205), 93–98. doi: 10.1126/science.1257530

Ilhan, Z. E., Łaniewski, P., Tonachio, A., and Herbst-Kralovetz, M. M. (2020). Members of prevotella genus distinctively modulate innate immune and barrier functions in a human three-dimensional endometrial epithelial cell model. *J. Infect. Dis.* 222 (12), 2082–2092. doi: 10.1093/infdis/jiaa324

Jan, R. L., Yeh, K. C., Hsieh, M. H., Lin, Y. L., Kao, H. F., Li, P. H., et al. (2012). Lactobacillus gasseri suppresses Th17 pro-inflammatory response and attenuates allergen-induced airway inflammation in a mouse model of allergic asthma. *Br. J. Nutr.* 108 (1), 130–139. doi: 10.1017/S0007114511005265

Jang, S.-E., Jeong, J.-J., Choi, S.-Y., Kim, H., Han, M. J., and Kim, D.-H. (2017). Lactobacillus rhamnosus HN001 and lactobacillus acidophilus la-14 attenuate gardnerella vaginalis-infected bacterial vaginosis in mice. *Nutrients* 9 (6), 531. doi: 10.3390/nu9060531

Jie, Z., Chen, C., Hao, L., Li, F., Song, L., Zhang, X., et al. (2021). Life history recorded in the vagino-cervical microbiome along with multi-omics. *Genomics Proteomics Bioinf.* 20 (2), 304–321. doi: 10.1016/j.gpb.2021.01.005

Joag, V., Obila, O., Gajer, P., Scott Milcah, C., Dizzell, S., Humphrys, M., et al. (2019). Impact of standard bacterial vaginosis treatment on the genital microbiota, immune milieu, and *ex vivo* human immunodeficiency virus susceptibility. *Clin. Infect. Dis.* 68 (10), 1675–1683. doi: 10.1093/cid/ciy762

Johnson, T. J., Clark, M. R., Albright, T. H., Nebeker, J. S., Tuitupou, A. L., Clark, J. T., et al. (2012). A 90-day tenofovir reservoir intravaginal ring for mucosal HIV prophylaxis. *Antimicrob. Agents Chemother.* 56 (12), 6272–6283. doi: 10.1128/AAC.01431-12

Kawahara, R., Fujii, T., Kukimoto, I., Nomura, H., Kawasaki, R., Nishio, E., et al. (2021). Changes to the cervicovaginal microbiota and cervical cytokine profile following surgery for cervical intraepithelial neoplasia. *Sci Rep* 11(1), 2156. doi: 10.1038/s41598-020-80176-6

Kero, K., Hieta, N., Kallonen, T., Ahtikoski, A., Laine, H. K., Rautava, J., et al. (2023). Optimal sampling and analysis methods for clinical diagnostics of vaginal microbiome. *Eur. J. Clin. Microbiol. Infect. Dis.* 42 (2), 201–208. doi: 10.1007/s10096-022-04545-x

Kim, S., Seo, H., Rahim, M. A., Lee, S., Kim, Y. S., and Song, H. Y. (2021). Changes in the microbiome of vaginal fluid after menopause in Korean women. *J. Microbiol. Biotechnol.* 31 (11), 1490–1500. doi: 10.4014/jmb.2106.06022

Kim, T. K., Thomas, S. M., Ho, M., Sharma, S., Reich, C. I., Frank, J. A., et al. (2009). Heterogeneity of vaginal microbial communities within individuals. *J. Clin. Microbiol.* 47 (4), 1181–1189. doi: 10.1128/JCM.00854-08

Klatt, N. R., Cheu, R., Birse, K., Zevin, A. S., Perner, M., Noël-Romas, L., et al. (2017). Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356 (6341), 938–945. doi: 10.1126/science.aai9383

Kobayashi, K., Matsunaga, K., Tsugami, Y., Wakasa, H., and Nishimura, T. (2021). IL-1β is a key inflammatory cytokine that weakens lactation-specific tight junctions of mammary epithelial cells. *Exp. Cell Res.* 409 (2), 112938. doi: 10.1016/j.yexcr.2021.112938

Kolev, Y., Uetake, H., Takagi, Y., and Sugihara, K. (2008). Lactate dehydrogenase-5 (LDH-5) expression in human gastric cancer: Association with hypoxia-inducible factor (HIF-1alpha) pathway, angiogenic factors production and poor prognosis. *Ann. Surg. Oncol.* 15 (8), 2336–2344. doi: 10.1245/s10434-008-9955-5

Kreisel, K. M., Weston, E. J., St Cyr, S. B., and Spicknall, I. H. (2021). Estimates of the prevalence and incidence of chlamydia and gonorrhea among US men and women 2018. *Sex Transm Dis.* 48 (4), 222–231. doi: 10.1097/OLQ.0000000000001382

Krog, M. C., Hugerth, L. W., Fransson, E., Bashir, Z., Nyboe Andersen, A., Edfeldt, G., et al. (2022). The healthy female microbiome across body sites: Effect of hormonal contraceptives and the menstrual cycle. *Hum. Reprod.* 37 (7), 1525–1543. doi: 10.1093/humrep/deac094

Kwok, L., Stapleton, A. E., Stamm, W. E., Hillier, S. L., Wobbe, C. L., and Gupta, K. (2006). Adherence of lactobacillus crispatus to vaginal epithelial cells from women with or without a history of recurrent urinary tract infection. *J. Urol.* 176 (5), 2050–2054. doi: 10.1016/j.juro.2006.07.014

Kyongo, J. K., Crucitti, T., Menten, J., Hardy, L., Cools, P., Michiels, J., et al. (2015). Cross-sectional analysis of selected genital tract immunological markers and molecular vaginal microbiota in Sub-Saharan African women, with relevance to HIV risk and prevention. *Clin. Vaccine Immunol.* 22 (5), 526–538. doi: 10.1128/CVI.00762-14

Łaniewski, P., and Herbst-Kralovetz, M. M. (2021). Bacterial vaginosis and health-associated bacteria modulate the immunometabolic landscape in 3D model of human cervix. *NPJ Biofilms Microbiomes* 7 (1), 88. doi: 10.1038/s41522-021-00259-8

Łaniewski, P., Barnes, D., Goulder, A., Cui, H., Roe Denise, J., Chase Dana, M., et al. (2018). Linking cervicovaginal immune signatures, HPV and microbiota composition in cervical carcinogenesis in non-Hispanic and Hispanic women. *Sci. Rep.* 8(1), 7593. doi: 10.1038/s41598-018-25879-7

Lebeau, A., Bruyere, D., Roncarati, P., Peixoto, P., Hervouet, E., Cobraiville, G., et al. (2022). HPV infection alters vaginal microbiome through down-regulating host mucosal innate peptides used by lactobacilli as amino acid sources. *Nat. Commun.* 13 (1), 1076. doi: 10.1038/s41467-022-28724-8

Leccese Terraf, M. C., Mendoza, L. M., Juárez Tomás, M. S., Silva, C., and Nader-Macías, M. E. F. (2014). Phenotypic surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal lactobacilli. *J. Appl. Microbiol.* 117 (6), 1761–1772. doi: 10.1111/jam.12642

Leppert, P. C. (1995). Anatomy and physiology of cervical ripening. *Clin. Obstet Gynecol* 38 (2), 267–279. doi: 10.1097/00003081-199506000-00009

Li, C., Jia, L., Yu, Y., and Jin, L. (2019). Lactic acid induced microRNA-744 enhances motility of SiHa cervical cancer cells through targeting ARHGAP5. *Chem. Biol. Interact.* 298 (undefined), 86–95. doi: 10.1016/j.cbi.2018.10.027

Li, B., and Sui, L. (2021). Metabolic reprogramming in cervical cancer and metabolomics perspectives. *Nutr. Metab. (Lond)* 18 (1), 93. doi: 10.1186/s12986-021-00615-7

Lin, F., Zhang, B., Shi, Q., Liang, J., Wang, X., Lian, X., et al. (2021). The conditioned medium of lactobacillus rhamnoides GG regulates Microglia/Macrophage polarization and improves functional recovery after spinal cord injury in rats. *BioMed. Res. Int.* 2021 (undefined), 3376496. doi: 10.1155/2021/3376496

Lithgow, K. V., Buchholz, V. C.H., Ku, E., Konschuh, S., D'Aubeterre, A., and Sycuro, L. K. (2022). Protease activities of vaginal porphyromonas species disrupt coagulation and extracellular matrix in the cervicovaginal niche. *NPJ Biofilms Microbiomes* 8 (1), 8. doi: 10.1038/s41522-022-00270-7

Liu, L., Chen, Yu., Chen, J.-L., Xu, H.-J., Zhan, H.-Y., Chen, Z., et al. (2021b). Integrated metagenomics and metabolomics analysis of third-trimester pregnant women with premature membrane rupture: A pilot study. *Ann. Transl. Med.* 9 (23), 1724. doi: 10.21037/atm-21-5539

Liu, C.-W., Su, B.-C., and Chen, J.-Y. (2021a). Gardnerella vaginalisTilapia piscidin 4 (TP4) reprograms M1 macrophages to M2 phenotypes in cell models of -induced vaginosis. *Front. Immunol.* 12 (undefined). doi: 10.3389/fimmu.2021.773013

López-Filloy, M., Cortez Flor, J., Gheit, T., Cruz Cruz, Y. O., Cruz-Talonia, F., Chávez-Torres, M., et al. (2022). Altered vaginal microbiota composition correlates with human papillomavirus and mucosal immune responses in women with symptomatic cervical ectopy. *Front. Cell. Infect. Microbiol.* 12 (undefined), 884272. doi: 10.3389/fcimb.2022.884272

Ma, L.-N., Huang, X.-B., Muyayalo, K. P., Mor, G., and Liao, A.-H. (2020). Lactic acid: A novel signaling molecule in early pregnancy? *Front. Immunol.* 11 (undefined). doi: 10.3389/fimmu.2020.00279

Maarsingh, J. D., Łaniewski, P., and Herbst-Kralovetz, M. M. (2022). Immunometabolic and potential tumor-promoting changes in 3D cervical cell models infected with bacterial vaginosis-associated bacteria. *Commun. Biol.* 5 (1), 725. doi: 10.1038/s42003-022-03681-6

Mahajan, G., Doherty, E., To, T., Sutherland, A., Grant, J., Junaid, A., et al. (2022). Vaginal microbiome-host interactions modeled in a human vagina-on-a-chip. *Microbiome* 10 (1), 201. doi: 10.1186/s40168-022-01400-1

Manzanares-Leal, G. L., Coronel-Martínez, J. A., Rodríguez-Morales, M., Rangel-Cuevas, Iván., Bustamante-Montes, L. P., Sandoval-Trujillo, H., et al. (2022). Preliminary identification of the aerobic cervicovaginal microbiota in Mexican women with cervical cancer as the first step towards metagenomic studies. *Front. Cell Infect. Microbiol.* 12 (undefined). doi: 10.3389/fcimb.2022.838491

Mastromarino, P., Di Pietro, M., Schiavoni, G., Nardis, C., Gentile, M., and Sessa, R. (2014). Effects of vaginal lactobacilli in chlamydia trachomatis infection. *Int. J. Med. Microbiol.* 304 (null), 654–661. doi: 10.1016/j.ijmm.2014.04.006

Mattei, V., Murugesan, S., Al Hashmi, M., Mathew, R., James, N., Singh, P., et al. (2019). Evaluation of methods for the extraction of microbial DNA from vaginal swabs used for microbiome studies. *Front. Cell Infect. Microbiol.* 9. doi: 10.3389/fcimb.2019.00197

McGregor, J. A., Lawellin, D., Franco-Buff, A., Todd, J. K., and Makowski, E. L. (1986). Protease production by microorganisms associated with reproductive tract infection. *Am. J. Obstet Gynecol* 154 (1), 109–114. doi: 10.1016/0002-9378(86)90404-7

McKenzie, R., Maarsingh, J. D., Łaniewski, P., and Herbst-Kralovetz, M. M. (2021). Immunometabolic analysis of mobiluncus mulieris and eggerthella sp. reveals novel insights into their pathogenic contributions to the hallmarks of bacterial vaginosis. *Front. Cell. infection Microbiol.* 11. doi: 10.3389/fcimb.2021.759697

Mirmonsef, P., Zariffard, M. R., Gilbert, D., Makinde, H., Landay, A. L., and Spear, G. T. (2012). Short-chain fatty acids induce pro-inflammatory cytokine production alone and in combination with toll-like receptor ligands. *Am. J. Reprod. Immunol.* 67 (5), 391–400. doi: 10.1111/j.1600-0897.2011.01089.x

Mitchell, C. M., Ma, N., Mitchell, A. J., Wu, M. C., Valint, D. J., Proll, S., et al. (2021a). Association between postmenopausal vulvovaginal discomfort, vaginal microbiota, and mucosal inflammation. *Am. J. Obstet Gynecol* 225 (2), 159.e151–159.e115. doi: 10.1016/j.ajog.2021.02.034

Mitchell, C., and Marrazzo, J. (2014). Bacterial vaginosis and the cervicovaginal immune response. *Am. J. Reprod. Immunol. (New York N.Y. 1989)* 71 (6), 555–563. doi: 10.1111/aji.12264

Mitchell, C. M., Srinivasan, S., Ma, N., Reed, S. D., Wu, M. C., Hoffman, N. G., et al. (2021b). Bacterial communities associated with abnormal Nugent score in postmenopausal versus premenopausal women. *J. Infect. Dis.* 223 (12), 2048–2052. doi: 10.1093/infdis/jiaa675

Mitra, A., MacIntyre, D. A., Lee, Y. S., Smith, A., Marchesi, J. R., Lehne, B, et al. (2015). Cervical intraepithelial neoplasia disease progression is associated with increased vaginal microbiome diversity. *Sci. Rep.* 5 (undefined), 16865. doi: 10.1038/srep16865

Mitra, A., MacIntyre, D. A., Ntritsos, G., Smith, A., Tsilidis, K. K., Marchesi, J. R., et al. (2020). The vaginal microbiota associates with the regression of untreated cervical intraepithelial neoplasia 2 lesions. *Nat. Commun.* 11 (1), 1999. doi: 10.1038/s41467-020-15856-y

Mohammadi, A., Bagherichimeh, S., Choi, Y., Fazel, A., Tevlin, E., Huibner, S., et al. (2022). Immune parameters of HIV susceptibility in the female genital tract before and after penile-vaginal sex. *Commun. Med. (Lond)* 2 (undefined), 60. doi: 10.1038/s43856-022-00122-7

Mohammadi, A., Bagherichimeh, S., Perry, M. C., Fazel, A., Tevlin, E., Huibner, S., et al. (2020). The impact of cervical cytobrush sampling on cervico-vaginal immune parameters and microbiota relevant to HIV susceptibility. *Sci. Rep.* 10 (1), 8514. doi: 10.1038/s41598-020-65544-6

Molano, M., Meijer, C. J.L.M., Weiderpass, E., Arslan, A., Posso, H., Franceschi, S., et al. (2005). The natural course of chlamydia trachomatis infection in asymptomatic Colombian women: A 5-year follow-up study. *J. Infect. Dis.* 191 (6), 907–916. doi: 10.1086/428287

Mollin, A., Katta, M., Sobel, J., and Akins, R. J. P. O. (2022). Association of key species of vaginal bacteria of recurrent bacterial vaginosis patients before and after oral metronidazole therapy with short- and long-term clinical outcomes. *PloS One* 17 (7), e0272012. doi: 10.1371/journal.pone.0272012

Moncla, B. J., Chappell, C. A., Debo, B. M., and Meyn, L. A. (2016). The effects of hormones and vaginal microflora on the glycome of the female genital tract: Cervical-vaginal fluid. *PloS One* 11 (7), e0158687. doi: 10.1371/journal.pone.0158687

Morré, S. A., van den Brule, A. J.C., Rozendaal, L., Boeke, A.J. P., Voorhorst Feja J.,, de Blok, S., et al. (2002). The natural course of asymptomatic chlamydia trachomatis infections: 45% clearance and no development of clinical PID after one-year follow-up. *Int. J. STD AIDS* null (undefined), 12–18. doi: 10.1258/095646202762226092

Moscicki, A. B., Shi, B., Huang, H., Barnard, E., and Li, H. (2020). Cervical-vaginal microbiome and associated cytokine profiles in a prospective study of HPV 16 acquisition, persistence, and clearance. *Front. Cell. infection Microbiol.* 10. doi: 10.3389/fcimb.2020.569022

Motevaseli, E., Azam, R., Akrami, S. M., Mazlomy, M., Saffari, M., Modarressi, M. H., et al. (2016). The effect of lactobacillus crispatus and lactobacillus rhamnosusCulture supernatants on expression of autophagy genes and HPV E6 and E7 oncogenes in the HeLa cell line. *Cell J.* 17 (4), 601–607. doi: 10.22074/cellj.2016.3833

Mott, P. D., Taylor, C. M., Lillis, R. A., Ardizzone, C. M., Albritton, H. L., Luo, M., et al. (2021). Differences in the genital microbiota in women who naturally clear chlamydia trachomatis infection compared to women who do not clear; a pilot study. *Front. Cell. infection Microbiol.* 11. doi: 10.3389/fcimb.2021.615770

Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., and Núñez, G. (2013). K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38 (6), 1142–1153. doi: 10.1016/j.immuni.2013.05.016

Munusamy, P. S., Thiruvengadam, K., Tellapragada, C., Ambikan, A. T., Narayanan, A., Kathirvel, S., et al. (2021). Deciphering the role of mucosal immune responses and the cervicovaginal microbiome in resistance to HIV infection in HIV-exposed seronegative (HESN) women. *Microbiol. Spectr.* 9 (2), e0047021. doi: 10.1128/Spectrum.00470-21

Mwenda, N., Nduati, R., Kosgey, M., and Kerich, G. (2021). Effect of bacterial vaginosis (BV)-HIV-1 Co-existence on maternal and infant health: A secondary data analysis. *Front. Pediatr.* 9 (undefined). doi: 10.3389/fped.2021.544192

Nagakubo, T., Nomura, N., and Toyofuku, M. (2019). Cracking open bacterial membrane vesicles. *Front. Microbiol.* 10 (undefined). doi: 10.3389/fmicb.2019.03026

Ñahui, P. R. A., Vanpouille, C., Laghi, L., Parolin, C., Melikov, K., Backlund, P., et al. (2019). Extracellular vesicles from symbiotic vaginal lactobacilli inhibit HIV-1 infection of human tissues. *Nat. Commun.* 10 (1), 5656. doi: 10.1038/s41467-019-13468-9

Ñahui, P. R. A., Zicari, S., Vanpouille, C., Vitali, B., and Margolis, L. (2017). LactobacillusVaginal inhibits HIV-1 replication in human tissues. *Front. Microbiol.* 8 (undefined). doi: 10.3389/fmicb.2017.00906

Ng, S., Chen, M., Kundu, S., Wang, X., Zhou, Z., Zheng, Z., et al. (2021). Large-Scale characterisation of the pregnancy vaginal microbiome and sialidase activity in a low-risk Chinese population. *NPJ biofilms microbiomes* 7 (1), 89. doi: 10.1038/s41522-021-00261-0

Nicolò, S., Tanturli, M., Mattiuz, G., Antonelli, A., Baccani, I., Bonaiuto, C., et al. (2021). Vaginal lactobacilli and vaginal dysbiosis-associated bacteria differently affect cervical epithelial and immune homeostasis and anti-viral defenses. *Int. J. Mol. Sci.* 22 (12), 6487. doi: 10.3390/ijms22126487

Nishiyama, K., Sugiyama, M., and Mukai, T. (2016). Adhesion properties of lactic acid bacteria on intestinal mucin. *Microorganisms* 4 (3), 34. doi: 10.3390/microorganisms4030034

Norenhag, J., Du, J., Olovsson, M., Verstraelen, H., Engstrand, L., and Brusselaers, N. (2020). The vaginal microbiota, human papillomavirus and cervical dysplasia: A systematic review and network meta-analysis. *BJOG* 127 (2), 171–180. doi: 10.1111/1471-0528.15854

Nunn, K. L., Wang, Y.-Y., Harit, D., Humphrys, M. S., Ma, B., Cone, R., et al. (2015). Enhanced trapping of HIV-1 by human cervicovaginal mucus is associated with lactobacillus crispatus-dominant microbiota. *mBio* 6 (5), e01084–e01015. doi: 10.1128/mBio.01084-15

Oerlemans, E., Ahannach, S., Wittouck, S., Dehay, E., De Boeck, I., Ballet, N., et al. (2022). Impacts of menstruation, community type, and an oral yeast probiotic on the vaginal microbiome. *mSphere* 7 (5), e0023922. doi: 10.1128/msphere.00239-22

Parada, V. D., de la Fuente, M. K., Landskron, G., González, MaríaJ., Quera, R., Dijkstra, G., et al. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front. Immunol.* 10 (undefined). doi: 10.3389/fimmu.2019.00277

Park, J., Kim, M., Kang, S. G., Jannasch, A. H., Cooper, B., Patterson, J., et al. (2015). Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. *Mucosal Immunol.* 8 (1), 80–93. doi: 10.1038/mi.2014.44

Parolin, C., Frisco, G., Foschi, C., Giordani, B., Salvo, M., Vitali, B., et al. (2018). Lactobacillus crispatus BC5 interferes with infectivity through integrin modulation in cervical cells. *Front. Microbiol.* 9 (undefined). doi: 10.3389/fmicb.2018.02630

Pattacini, L., Woodward, D. A., Czartoski, J., Mair, F., Presnell, S., Hughes, S. M., et al. (2019). A pro-inflammatory CD8+ T-cell subset patrols the cervicovaginal tract. *Mucosal Immunol.* 12 (5), 1118–1129. doi: 10.1038/s41385-019-0186-9

Plisko, O., Zodzika, J., Jermakova, I., Pcolkina, K., Prusakevica, A., Liepniece-Karele, I., et al. (2021). Aerobic vaginitis-underestimated risk factor for cervical intraepithelial neoplasia. *Diagnostics (Basel)* 11 (1), 97. doi: 10.3390/diagnostics11010097

Pudney, J., Quayle, A. J., and Anderson, D. J. (2005). Immunological microenvironments in the human vagina and cervix: Mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol. Reprod.* 73 (6), 1253–1263. doi: 10.1095/biolreprod.105.043133

Radtke, A. L., Quayle, A. J., and Herbst-Kralovetz, M. M. (2012). Microbial products alter the expression of membrane-associated mucin and antimicrobial peptides in a three-dimensional human endocervical epithelial cell model. *Biol. Reprod.* 87 (6), 132. doi: 10.1095/biolreprod.112.103366

Raimondi, S., Candeliere, F., Amaretti, A., Foschi, C., Morselli, S., Gaspari, V., et al. (2021). Chlamydia trachomatis vaginal and anal microbiome during infections. *Pathogens* 10 (10), 1347. doi: 10.3390/pathogens10101347

Rajeeve, K., Vollmuth, N., Janaki-Raman, S., Wulff, T. F., Baluapuri, A., Dejure, F. R., et al. (2020). Reprogramming of host glutamine metabolism during chlamydia trachomatis infection and its key role in peptidoglycan synthesis. *Nat. Microbiol.* 5 (11), 1390–1402. doi: 10.1038/s41564-020-0762-5

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. United States America* 108 Suppl 1 (Suppl 1), 4680–4687. doi: 10.1073/pnas.1002611107

Read, C. P., Word, R.A., Ruscheinsky, M. A., Timmons, B. C., and Mahendroo, M. S. (2007). Cervical remodeling during pregnancy and parturition: Molecular characterization of the softening phase in mice. *Reproduction* 134 (2), 327–340. doi: 10.1530/REP-07-0032

Rizzo, A., Fiorentino, M., Buommino, E., Donnarumma, G., Losacco, A., and Bevilacqua, N. (2015). Lactobacillus crispatus mediates anti-inflammatory cytokine interleukin-10 induction in response to chlamydia trachomatis infection *in vitro*. *Int. J. Med. Microbiol.* 305 (8), 815–827. doi: 10.1016/j.ijmm.2015.07.005

Robinson, L. S., Schwebke, J., Lewis, W. G., and Lewis, A. L. (2019). Gardnerella vaginalisIdentification and characterization of NanH2 and NanH3, enzymes responsible for sialidase activity in the vaginal bacterium. *J. Biol. Chem.* 294 (14), 5230–5245. doi: 10.1074/jbc.RA118.006221

Rodriguez-Garcia, M., Barr, F. D., Crist, S. G., Fahey, J. V., and Wira, C. R. (2014). Phenotype and susceptibility to HIV infection of CD4+ Th17 cells in the human female reproductive tract. *Mucosal Immunol.* 7 (6), 1375–1385. doi: 10.1038/mi.2014.26

Rodriguez-Garcia, M., Fortier, J. M., Barr, F. D., and Wira, C. R. (2018). Aging impacts CD103 CD8 T cell presence and induction by dendritic cells in the genital tract. *Aging Cell* 17 (3), e12733. doi: 10.1111/acel.12733

Rodriguez-Garcia, M., Shen, Z., Fortier, J. M., and Wira, C. R. (2020). Differential cytotoxic function of resident and non-resident CD8+ T cells in the human female reproductive tract before and after menopause. *Front. Immunol.* 11 (undefined). doi: 10.3389/fimmu.2020.01096

Rosato, P. C., Beura, L. K., and Masopust, D. (2017). Tissue resident memory T cells and viral immunity. *Curr. Opin. Virol.* 22, 44–50. doi: 10.1016/j.coviro.2016.11.011

Sahu, U., and Khare, P. (2021). Role of interleukin-17 in human papillomavirus infection and associated malignancies. *Microb. Pathog.* 161 (null), 105294. doi: 10.1016/j.micpath.2021.105294

Salliss, M. E., Maarsingh, J. D., Garza, C., Łaniewski, P., and Herbst-Kralovetz, M. M. (2021). Veillonellaceae family members uniquely alter the cervical metabolic microenvironment in a human three-dimensional epithelial model. *NPJ biofilms microbiomes* 7 (1), 57. doi: 10.1038/s41522-021-00229-0

Sathaliyawala, T., Kubota, M., Yudanin, N., Turner, D., Camp, P., Thome, J. J.C., et al. (2013). Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* 38 (1), 187–197. doi: 10.1016/j.immuni.2012.09.020

Schenkel, J. M., Fraser, K. A., Beura, L. K., Pauken, K. E., Vezys, V., and Masopust, D. (2014). T Cell memory. resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* 346 (6205), 98–101. doi: 10.1126/science.1254536

Schnaars, V., Dörries, M., Hutchins, M., Wöhlbrand, L., and Rabus, R. (2018). What's the difference? 2D DIGE image analysis by DeCyderTM versus SameSpotsTM. *J. Mol. Microbiol. Biotechnol.* 28 (3), 128–136. doi: 10.1159/000494083

Scillato, M., Spitale, A., Mongelli, G., Privitera, G. F., Mangano, K., Cianci, A., et al. (2021). Antimicrobial properties of lactobacillus cell-free supernatants against multidrug-resistant urogenital pathogens. *Microbiologyopen* 10 (2), e1173. doi: 10.1002/mbo3.1173

Serebrenik, J., Wang, T, Hunte, R., Srinivasan, S., McWalters, J., Tharp Gregory, K., et al. (2021). Differences in vaginal microbiota, host transcriptome, and proteins in women with bacterial vaginosis are associated with metronidazole treatment response. *J. Infect. Dis.* 224 (12), 2094–2104. doi: 10.1093/infdis/jiab266

Sessa, R., Di Pietro, M., Filardo, S., Bressan, A., Rosa, L., Cutone, A., et al. (2017). Effect of bovine lactoferrin on chlamydia trachomatis infection and inflammation. *Biochem. Cell Biol.* 95 (1), 34–40. doi: 10.1139/bcb-2016-0049

Shannon, B., Gajer, P., Yi, T. J., Ma, B., Humphrys, M. S., Thomas-Pavanel, J., et al. (2017). Distinct effects of the cervicovaginal microbiota and herpes simplex type 2 infection on female genital tract immunology. *J. Infect. Dis.* 215 (9), 1366–1375. doi: 10.1093/infdis/jix088

Shardell, M., Gravitt, P. E., Burke, A. E., Ravel, J., and Brotman, R. M. (2021). Association of vaginal microbiota with signs and symptoms of the genitourinary syndrome of menopause across reproductive stages. *J. Gerontol A Biol. Sci. Med. Sci.* 76 (9), 1542–1550. doi: 10.1093/gerona/glab120

Short, C.-E. S., Brown, R. G., Quinlan, R., Lee, Y. S., Smith, A., Marchesi, J. R., et al. (2020). Lactobacillus-depleted vaginal microbiota in pregnant women living with HIV-1 infection are associated with increased local inflammation and preterm birth. *Front. Cell Infect. Microbiol.* 10 (undefined). doi: 10.3389/fcimb.2020.596917

Short, C.-E. S., Quinlan, R. A., Wang, X., Preda, V. G., Smith, A., Marchesi, J. R., et al. (2021). Vaginal microbiota, genital inflammation and extracellular matrix remodelling collagenase: MMP-9 in pregnant women with HIV, a potential preterm birth mechanism warranting further exploration. *Front. Cell Infect. Microbiol.* 11 (undefined). doi: 10.3389/fcimb.2021.750103

Shukair, S. A., Allen, S. A., Cianci, G. C., Stieh, D. J., Anderson, M. R., Baig, S. M., et al. (2013). Human cervicovaginal mucus contains an activity that hinders HIV-1 movement. *Mucosal Immunol.* 6(2), 427–34. doi: 10.1038/mi.2012.87.

Sierra, L.-J., Brown, A. G., Barilá, G. O., Anton, L., Barnum, C. E., Shetye, S. S., et al. (2018). Colonization of the cervicovaginal space with gardnerella vaginalis leads to local inflammation and cervical remodeling in pregnant mice. *PloS One* 13 (1), e0191524. doi: 10.1371/journal.pone.0191524

Sirichoat, A., Sankuntaw, N., Engchanil, C., Buppasiri, P., Faksri, K., Namwat, W., et al. (2021). Comparison of different hypervariable regions of 16S rRNA for taxonomic profiling of vaginal microbiota using next-generation sequencing. *Arch. Microbiol.* 203 (3), 1159–1166. doi: 10.1007/s00203-020-02114-4

Smith, S. B., and Ravel, J. (2017). The vaginal microbiota, host defence and reproductive physiology. *J. Physiol.* 595 (2), 451–463. doi: 10.1113/JP271694

Sobia, P., Pillay, T., Liebenberg, L. J.P., Sivro, A., Mansoor, L. E., Osman, F., et al. (2021). Higher mucosal antibody concentrations in women with genital tract inflammation. *Sci. Rep.* 11 (1), 23514. doi: 10.1038/s41598-021-02954-0

Song, S. D., Acharya, K. D., Zhu, J. E., Deveney, C. M., Walther-Antonio, M. R. S., Tetel, M. J., et al. (2020). Daily vaginal microbiota fluctuations associated with natural hormonal cycle, contraceptives, diet, and exercise. *mSphere* 5 (4), e00593–20. doi: 10.1128/mSphere.00593-20

Srinivasan, S., Morgan, M. T., Fiedler, T. L., Djukovic, D., Hoffman, N. G., Raftery, D., et al. (2015). Metabolic signatures of bacterial vaginosis. *mBio* 6 (2), e00204–15. doi: 10.1128/mBio.00204-15

Stallmann, S., and Hegemann, J. H. (2016). The chlamydia trachomatis Ctad1 invasin exploits the human integrin β1 receptor for host cell entry. *Cell Microbiol.* 18 (5), 761–775. doi: 10.1111/cmi.12549

Sungur, T., Aslim, B., Karaaslan, C., and Aktas, B. (2017). Impact of exopolysaccharides (EPSs) of lactobacillus gasseri strains isolated from human vagina on cervical tumor cells (HeLa). *Anaerobe* 47, 137–144. doi: 10.1016/j.anaerobe.2017.05.013

Takada, K., Komine-Aizawa, S., Kuramochi, T., Ito, S., Trinh, Q. D., Pham Ngan, T. K., et al. (2018). Lactobacillus crispatus accelerates re-epithelialization in vaginal epithelial cell line MS74. *Am. J. Reprod. Immunol.* 80 (3), e13027. doi: 10.1111/aji.13027

Taku, O., Onywera, H., Mbulawa, Z. Z. A., Businge, C. B., Meiring, T. L., and Williamson, A. L. (2022). Molecular identification of cervical microbes in HIV-negative and HIV-positive women in an African setting using a customized bacterial vaginosis microbial DNA quantitative PCR (qPCR) array. *Microbiol. Spectr.* 10 (3), e0222921. doi: 10.1128/spectrum.02229-21

Tamarelle, J., Ma, B., Gajer, P., Humphrys, M. S., Terplan, M., Mark, K. S., et al. (2020). Nonoptimal vaginal microbiota after azithromycin treatment for chlamydia trachomatis infection. *J. Infect. Dis.* 221 (4), 627–635. doi: 10.1093/infdis/jiz499

Tamarelle, J., Thiébaut, A. C. M., de Barbeyrac, B., Bébéar, C., Ravel, J., and Delarocque-Astagneau, E. (2019). The vaginal microbiota and its association with human papillomavirus, chlamydia trachomatis, neisseria gonorrhoeae and mycoplasma genitalium infections: A systematic review and meta-analysis. *Clin. Microbiol. Infect.* 25 (1), 35–47. doi: 10.1016/j.cmi.2018.04.019

Taneva, E., Sinclair, S., Mesquita, P. Mm., Weinrick, B., Cameron, S. A., Cheshenko, N., et al. (2018). Vaginal microbiome modulates topical antiretroviral drug pharmacokinetics. *JCI Insight* 3 (13), e99545. doi: 10.1172/jci.insight.99545

Tantengco, O. A. G., Richardson, L. S., Medina, P. M. B., Han, A., and Menon, R. (2021a). Organ-on-chip of the cervical epithelial layer: A platform to study normal and pathological cellular remodeling of the cervix. *FASEB J.* 35 (4), e21463. doi: 10.1096/fj.202002590RRR

Tantengco, O. A. G., Vink, J., Medina, P. M. B., and Menon, R. (2021b). Oxidative stress promotes cellular damages in the cervix: Implications for normal and pathologic cervical function in human pregnancy†. *Biol. Reprod.* 105 (1), 204–216. doi: 10.1093/biolre/ioab058

Theodoridis, G., Gika, H., Want, E., and Wilson, I. (2012). Liquid chromatography-mass spectrometry based global metabolite profiling: A review. *Anal. Chim. Acta* 711, 7–16. doi: 10.1016/j.aca.2011.09.042

Thurman, A. R., Kimble, T., Herold, B., Mesquita, P. M.M., Fichorova, R. N., Dawood, H. Y., et al. (2015). Bacterial vaginosis and subclinical markers of genital tract inflammation and mucosal immunity. *AIDS Res. Hum. Retroviruses* 31 (11), 1139–1152. doi: 10.1089/aid.2015.0006

Thurman, A. R., Ravel, J., Gajer, P., Marzinke, M. A., Ouattara, L. A., Jacot, T., et al. (2022). Vaginal microbiota and mucosal pharmacokinetics of tenofovir in healthy women using a 90-day Tenofovir/Levonorgestrel vaginal ring. *Front. Cell Infect. Microbiol.* 12 (undefined). doi: 10.3389/fcimb.2022.799501

Timmons, B. C., Mitchell, S. M., Gilpin, C., and Mahendroo, M. S. (2007). Dynamic changes in the cervical epithelial tight junction complex and differentiation occur during cervical ripening and parturition. *Endocrinology* 148 (3), 1278–1287. doi: 10.1210/en.2006-0851

Trifonova, R. T., Lieberman, J., and van Baarle, D. (2014). Distribution of immune cells in the human cervix and implications for HIV transmission. *Am. J. Reprod. Immunol.* 71 (3), 252–264. doi: 10.1111/aji.12198

Tyssen, D., Wang, Y.-Y., Hayward, J. A., Agius, P. A., DeLong, K., Aldunate, M., et al. (2018). Anti-HIV-1 activity of lactic acid in human cervicovaginal fluid. *mSphere* 3 (4), e00055–18. doi: 10.1128/mSphere.00055-18

Ulcova-Gallova, Z. (2010). Immunological and physicochemical properties of cervical ovulatory mucus. *J. Reprod. Immunol.* 86 (2), 115–121. doi: 10.1016/j.jri.2010.07.002

Usyk, M., Schlecht, N. F., Pickering, S., Williams, L., Sollecito, C. C., Gradissimo, A., et al. (2022). molBV reveals immune landscape of bacterial vaginosis and predicts human papillomavirus infection natural history. *Nat. Commun.* 13 (1), 233. doi: 10.1038/s41467-021-27628-3

Usyk, M., Zolnik, C. P., Castle, P. E., Porras, C., Herrero, R., Gradissimo, A., et al. (2020). Cervicovaginal microbiome and natural history of HPV in a longitudinal study. *PloS Pathog.* 16 (3), e1008376. doi: 10.1371/journal.ppat.1008376

Vandergeeten, C., Fromentin, R., and Chomont, N. (2012). The role of cytokines in the establishment, persistence and eradication of the HIV reservoir. *Cytokine Growth factor Rev.* 23 (4-5), 143–149. doi: 10.1016/j.cytogfr.2012.05.001

van der Veer, C., Bruisten, S. M., van der Helm, J. J., de Vries, H. J.C., and van Houdt, R. (2017). The cervicovaginal microbiota in women notified for chlamydia trachomatis infection: A case-control study at the sexually transmitted infection outpatient clinic in Amsterdam, the Netherlands. *Clin. Infect. Dis.* 64 (1), 24–31. doi: 10.1093/cid/ciw586

van Houdt, R., Ma, B., Bruisten, S. M., Speksnijder, A. G.C.L., Ravel, J., and de Vries, H. J.C. (2018). Lactobacillus iners-dominated vaginal microbiota is associated with increased susceptibility to infection in Dutch women: A case-control study. *Sex Transm Infect.* 94 (2), 117–123. doi: 10.1136/sextrans-2017-053133

van Teijlingen, N. H., Helgers, L. C., Zijlstra-Willems, E. M., van Hamme, J. L., Ribeiro, C. M.S., Strijbis, K., et al. (2020). Vaginal dysbiosis associated-bacteria megasphaera elsdenii and prevotella timonensis induce immune activation via dendritic cells. *J. Reprod. Immunol.* 138 (undefined), 103085. doi: 10.1016/j.jri.2020.103085

Vick, E. J., Park, H. S., Huff, K. A., Brooks, K. M., Farone, A. L., and Farone, M. B. (2014). Gardnerella vaginalis triggers NLRP3 inflammasome recruitment in THP-1 monocytes. *J. Reprod. Immunol.* 106, 67–75. doi: 10.1016/j.jri.2014.08.005

Vinolo, M., Rodrigues, H., Hatanaka, E., Hebeda, C., Farsky, S., and Curi, R. (2009). Short-chain fatty acids stimulate the migration of neutrophils to inflammatory sites. *Clin. Sci. (Lond)* 117 (9), 331–338. doi: 10.1042/CS20080642

Virtanen, S., Kalliala, I., Nieminen, P., and Salonen, A. (2017). Comparative analysis of vaginal microbiota sampling using 16S rRNA gene analysis. *PloS One* 12 (7), e0181477. doi: 10.1371/journal.pone.0181477

Vitali, B., Cruciani, F., Picone, G., Parolin, C., Donders, G., and Laghi, L. (2015). Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 34 (12), 2367–2376. doi: 10.1007/s10096-015-2490-y

Vollmuth, N., Schlicker, L., Guo, Y., Hovhannisyan, P., Janaki-Raman, S., Kurmasheva, N., et al. (2022). C-myc plays a key role in IFN-γ-induced persistence of chlamydia trachomatis. *Elife* 11, e76721. doi: 10.7554/eLife.76721

Wagner, W., Ciszewski, W. M., and Kania, K. D. (2015). L- and d-lactate enhance DNA repair and modulate the resistance of cervical carcinoma cells to anticancer drugs *via* histone deacetylase inhibition and hydroxycarboxylic acid receptor 1 activation. *Cell Commun. Signal* 13 (undefined), 36. doi: 10.1186/s12964-015-0114-x

Wagner, W., Kania, K. D., and Ciszewski, W. M. (2017). Stimulation of lactate receptor (HCAR1) affects cellular DNA repair capacity. *DNA Repair (Amst)* 52 (undefined), 49–58. doi: 10.1016/j.dnarep.2017.02.007

Wagner, W., Sobierajska, K., Kania, K. D., Paradowska, E., and Ciszewski, W. Michał. (2021). Lactate suppresses retroviral transduction in cervical epithelial cells through DNA-PKcs modulation. *Int. J. Mol. Sci.* 22 (24), 13194. doi: 10.3390/ijms222413194

Walenta, S., Wetterling, M., Lehrke, M., Schwickert, G., Sundfør, K., Rofstad, E. K., et al. (2000). High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. *Cancer Res.* 60 (4), 916–921. doi: 10.1016/j.diagmicrobio.2019.114981

Wang, X.-H., Song, T.-Z., Zheng, H.-Y., Li, Y.-H., and Zheng, Y.-T. (2021b). Jejunal epithelial barrier disruption triggered by reactive oxygen species in early SIV infected rhesus macaques. *Free Radic. Biol. Med.* 177 (undefined), 143–155. doi: 10.1016/j.freeradbiomed.2021.10.026

Wang, R., Zhou, G., Wu, L., Huang, X., Li, Y., Luo, B., et al. (2021a). *in vitro*The microbial composition of lower genital tract may affect the outcome of fertilization-embryo transfer. *Front. Microbiol.* 12 (undefined). doi: 10.3389/fmicb.2021.729744

Wang, C., Fan, A., Li, H., Yan, Y., Qi, W., Wang, Y., et al. (2020). Vaginal bacterial profiles of aerobic vaginitis: A case-control study. *Diagn Microbiol Infect Dis* 96(4), 114981. doi: 10.1016/j.diagmicrobio.2019.114981

Warburg, O. (1956). On the origin of cancer cells. Science 123(3191), 309–14. doi: 10.1126/science.123.3191.309

Watson, E., and Reid, G. (2018). Metabolomics as a clinical testing method for the diagnosis of vaginal dysbiosis. *Am. J. Reprod. Immunol.* 80 (2), e12979. doi: 10.1111/aji.12979

White, H. D., Crassi, K. M., Givan, A. L., Stern, J. E., Gonzalez, J. L., Memoli, V. A., et al. (1997). CD3+ CD8+ CTL activity within the human female reproductive tract: Influence of stage of the menstrual cycle and menopause. *J. Immunol.* 158 (6), 3017–3027. doi: 10.4049/jimmunol.158.6.3017

Wiesenfeld, H. C., Hillier, S. L., Krohn, M. A., Landers, D. V., and Sweet, R. L. (2003). Bacterial vaginosis is a strong predictor of neisseria gonorrhoeae and chlamydia trachomatis infection. *Clin. Infect. Dis.* 36 (5), 663–668. doi: 10.1086/367658

Wiggins, R., Millar, M. R., Soothill, P. W., Hicks, S. J., and Corfield, A. P. (2002). Application of a novel human cervical mucin-based assay demonstrates the absence of increased mucinase activity in bacterial vaginosis. *Int. J. STD AIDS* 13 (11), 755–760. doi: 10.1258/095646202320753709

Wira, C. R., Fahey, J. V., Ghosh, M., Patel, M. V., Hickey, D. K., and Ochiel, D. O. (2010). Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *Am. J. Reprod. Immunol.* 63 (6), 544–565. doi: 10.1111/j.1600-0897.2010.00842.x

Witkin, S. S., Mendes-Soares, H., Linhares, I. M., Jayaram, A., Ledger, W. J., and Forney, L. J. (2013). Influence of vaginal bacteria and d- and l-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: Implications for protection against upper genital tract infections. *mBio* 4 (4), e00460–e00413. doi: 10.1128/mBio.00460-13

Xiang, N., Yin, T., and Chen, T. (2021). Gardnerella vaginalis induces NLRP3 inflammasome-mediated pyroptosis in macrophages and THP-1 monocytes. *Exp. Ther. Med.* 22 (4), 1174. doi: 10.3892/etm.2021.10609

Xiao, B., Qin, H., Mi, L., and Zhang, D. (2022). Correlation analysis of vaginal microbiome changes and bacterial vaginosis plus vulvovaginal candidiasis mixed vaginitis prognosis. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.860589

Xiao, B., Wu, C., Song, W., Niu, X., Qin, N., Liu, Z., et al. (2019). Association analysis on recurrence of bacterial vaginosis revealed microbes and clinical variables important for treatment outcome. *Front. Cell Infect. Microbiol.* 9. doi: 10.3389/fcimb.2019.00189

Xue, J., Wang, Y., Chen, C., Zhu, X., Zhu, H., and Hu, Y. (2018). Effects of Th17 cells and IL-17 in the progression of cervical carcinogenesis with high-risk human papillomavirus infection. *Cancer Med.* 7 (2), 297–306. doi: 10.1002/cam4.1279

Yamamoto, N., Kan-O, K., Tatsuta, M., Ishii, Y., Ogawa, T., Shinozaki, S., et al. (2021). Incense smoke-induced oxidative stress disrupts tight junctions and bronchial epithelial barrier integrity and induces airway hyperresponsiveness in mouse lungs. *Sci. Rep.* 11 (1), 7222. doi: 10.1038/s41598-021-86745-7

Yellon, S. M. (2019). Immunobiology of cervix ripening. *Front. Immunol.* 10 (undefined). doi: 10.3389/fimmu.2019.03156

Zariffard, M.R., Novak, R. M., Lurain, N., Sha, B. E., Graham, P., and Spear, G. T. (2005). Induction of tumor necrosis factor- alpha secretion and toll-like receptor 2 and 4 mRNA expression by genital mucosal fluids from women with bacterial vaginosis. *J. Infect. Dis.* 191 (11), 1913–1921. doi: 10.1086/429922

Zevin, A. S., Xie, I. Y., Birse, K., Arnold, K., Romas, L., Westmacott, G., et al. (2016). Microbiome composition and function drives wound-healing impairment in the female genital tract. *PloS Pathog.* 12 (9), e1005889. doi: 10.1371/journal.ppat.1005889

Zhang, Z., Li, T., Zhang, D., Zong, X., Bai, H., Bi, H., et al. (2021). Distinction between vaginal and cervical microbiota in high-risk human papilloma virus-infected women in China. *BMC Microbiol.* 21 (1), 90. doi: 10.1186/s12866-021-02152-y

Zhang, Y., Xu, X., Yu, L., Shi, X., Min, M., Xiong, L., et al. (2022). Vaginal microbiota changes caused by HPV infection in Chinese women. *Front. Cell Infect. Microbiol.* 12 (undefined). doi: 10.3389/fcimb.2022.814668

Zhou, R., Lu, J., Wang, J., and Xiao, B. (2022). Vaginal lactobacillus iners abundance is associated with outcome in antibiotic treatment of bacterial vaginosis and capable of inhibiting gardnerella. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.1033431

Ziklo, N., Huston, W. M., Taing, K., Katouli, M., and Timms, P. (2016). *In vitro* rescue of genital strains of chlamydia trachomatis from interferon-γ and tryptophan depletion with indole-positive, but not indole-negative prevotella spp. *BMC Microbiol.* 16 (1), 286. doi: 10.1186/s12866-016-0903-4

Ziklo, N., Huston, W. M., Taing, K., and Timms, P. (2019). High expression of IDO1 and TGF-β1 during recurrence and post infection clearance with chlamydia trachomatis, are independent of host IFN-γ response. *BMC Infect. Dis.* 19 (1), 218. doi: 10.1186/s12879-019-3843-4

Ziklo, N., Vidgen, M. E., Taing, K., Huston, W. M., and Timms, P. (2018). Dysbiosis of the vaginal microbiota and higher vaginal Kynurenine/Tryptophan ratio reveals an association with chlamydia trachomatis genital infections. *Front. Cell Infect. Microbiol.* 8. doi: 10.3389/fcimb.2018.00001

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EDITED BY
Zhangran Chen,
Shenzhen Wedge Microbiology Research Co.LTD, China

REVIEWED BY
Li-Shang Dai,
Wenzhou Medical University, China
Kun Li,
Nanjing Agricultural University, China

*CORRESPONDENCE
Heqing Shen
hqshen@xmu.edu.cn
Xueqin Zhang
wind459@126.com

†These authors have contributed equally to this work

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Maternal vaginal fluids play a major role in the colonization of the neonatal intestinal microbiota

Jingxian Xie[1†], Chen Tang[2,3†], Shouqiang Hong[3†], Yuntian Xin[4], Jie Zhang[3], Yi Lin[3], Lindong Mao[3], Yunshan Xiao[1], Quanfeng Wu[1], Xueqin Zhang[1*] and Heqing Shen[1,3*]

[1]Department of Obstetrics, Women and Children's Hospital, School of Medicine, Xiamen University, Xiamen, Fujian, China, [2]School of Life Sciences, Xiamen University, Xiamen, Fujian, China, [3]State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, Fujian, China, [4]Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, Fujian, China

Background: Caesarean section (CS) is associated with newborns' health risks due to the blocking of microbiome transfer. The gut microbiota of CS-born babies was different from those born vaginally, which may be attributed to reduced exposure to maternal vaginal microbes during labour. To understand the microbial transfer and reduce CS disadvantages, the effect of vaginal microbiota exposure on infant gut microbiota composition was evaluated using 16s rDNA sequencing-based techniques.

Results: Pregnant women were recruited in the Women and Children's Hospital, School of Medicine, Xiamen University from June 1st to August 15th, 2017. Maternal faeces (n = 26), maternal vaginal fluids (n = 26), and neonatal transitional stools (n = 26) were collected, while the participants underwent natural delivery (ND) (n = 6), CS (n = 4) and CS with the intervention of vaginal seedings (I) (n = 16). 26 mothers with the median age 26.50 (25.00-27.25) years showed no substantial clinical differences. The newborns' gut microbiota altered among ND, CS and I, and clustered into two groups (PERMANOVA $P = 0.001$). Microbial composition of ND babies shared more features with maternal vaginal samples (PERMANOVA $P = 0.065$), while the microbiota structure of ND babies was obviously different from that of sample of maternal faeces. The genus *Bacteroides* in CS-born babies with intervention approached to vaginal-born neonates, compared with CS-born neonates without intervention.

Conclusions: Neonatal gut microbiota was dependent on the delivery mode. And the gut microbiota CS newborns with vaginal seeding shared more features with those of ND babies, which hinted the aberrant gut microbiota composition initiated by CS might be partly mitigated by maternal vaginal microbiota exposure.

KEYWORDS

caesarean section, gut microbiome, newborn, treatment, vaginal fluid, transitional stool

Background

Caesarean section (CS) is a common obstetric surgical procedure entailing the incision of a woman's abdomen/uterus to deliver their offspring(s) (Seidu et al., 2020) with the intent to increase the chances of successful childbirth and to protect the life security of both the mother and the newborn (Betran et al., 2016; Cegolon et al., 2020). Over the past two to three decades, global CS surgery rates have been growing steadily but rapidly for women of all ages, races, and medical conditions. According to the World Health Organization (WHO), an estimated 21.1% of births occurred by CS in 2015, which was almost twice than that in 2000 (12.1%), and the rates were even higher in certain developed countries and regions (Ekstrom et al., 2020; Betran et al., 2018; Boerma et al., 2018; Wells et al., 2019). Similar patterns could also be observed in China, in which 28.8% and 34.9% of babies were delivered by CS in 2008 and 2014, respectively (Li et al., 2017).

However, prior publications have demonstrated that CS was associated with adverse short- or long-term effects on newborns, including the dysplasia of the immune system, infections, allergies, and inflammatory disorders (Mueller et al., 2015a; Mueller et al., 2017a; Rusconi et al., 2017). The conventional view concerning this correlation was that CS newborns were subjected to different hormonal, physical (mechanical forces), bacterial, and therapeutic conditions (Sandall et al., 2018). Among these conditions, differences in microbial colonization induced by delivery mode were thought to be one of the determining factors (Martin et al., 2016; Mueller et al., 2017a). The gut microbiota has been increasingly recognized as an important contributor to human health, especially for infants, whose maturity of immune system and overall physiology are influenced the gut microbiota (Korpela et al., 2018). Altered colonization of the gut microbiota in CS-born babies may partially account for the increased risk of adverse health conditions (Butler É et al., 2020). The sterility of the womb was widely accepted for many years (Perez-Muñoz et al., 2017). Although it is controversial about whether the presence of bacterial DNA contradicts the "sterile womb paradigm", it does not demonstrate the presence of a living microbiota (Ferretti et al., 2018; He et al., 2020). The prevailing view held that human fetal environment is sterile and the neonate's microbiome is acquired during and after birth (Perez-Muñoz et al., 2017; Walter and Hornef, 2021). Fetal development is an important period for human beings, and the extent to which modern practices, like CS, alter the microbial composition are still not completely understood (Dominguez-Bello et al., 2019). The exposure of newborns to the maternal vaginal microbiota might be interrupted by CS. Unlikely to vaginally delivered newborns, who are first exposed to a wide array of microbes during labour *via* direct contact with the birth canal, CS newborns are initially contacted with microbes from the delivery room and the mothers' skin (Chu et al., 2017). In addition, prophylactic antibiotics administered before or during caesarean surgery may also result in the failure of newborns to acquire the normal microbial inoculum (Azad et al., 2016; Stearns et al., 2017). Thus, vaginal fluids exposure (also known as "vaginal seedings") on CS-born babies shortly after birth is a potential way for reducing differences in the gut microbiota between CS-born and vaginal-born neonates (Butler É et al., 2020; The American College of Obstetricians and Gynecologists, 2017). A better understanding about such practice on infant gut microbiota composition are required to break this cycle. Moreover, exploring an effective intervention measure which is simple, convenient, and cost effective to compensate for the differences in gut microbiota composition of CS births could be a prospective application. Here, we used transitional stool as the biological sample in our present work, which was defined as the faeces excreted by newborns between 36 h and 72 h after birth (Mueller et al., 2017b). It represents the transitional state of meconium and may vary quickly *via* early-life microbial colonization in the surrounding environment. Only a few studies have aimed to explore the microbial community in transitional faeces.

Via the swabbing of CS newborns with their mothers' vaginal fluids, we aimed to explore the effect of vaginal microbiota exposure on infant gut microbiota composition in this study. By analyzing the pros and cons of the treatment through a population-based intervention study in Xiamen, PR China, we assess its effectiveness and performance in altering the microbes in newborns' transitional stools.

Methods

The recruitment of subjects

This intervention study invited pregnant women who received antenatal care at the Women and Children's Hospital, School of Medicine, Xiamen University, from June 1st, 2017 to August 15th, 2017. The inclusion criteria were briefly listed as follows: 1) gestational age between 37 and 42 weeks; 2) pregnant women who had regular prenatal visits and for whom all clinical data could be obtained; and 3) newborns who were full-term deliveries. Pregnant women with the following complications were excluded: 1) infectious diseases caused by bacteria, viruses, or parasites; 2) inflammatory diseases (e.g., ankylosing spondylitis); 3) metabolic diseases such as diabetes mellitus; 4) pregnancy-associated illnesses including preeclampsia and gestational hypertension; 5) reproductive system disorders (e.g., an ovarian cyst); 6) abnormal pregnancy state (e.g., preterm birth); 7) genetic diseases such as thalassemia; and 8) tumors, including pituitary adenomas and uterine fibroids. The specific analytical protocol of this study is shown in Figure S1. The basic information of the pregnant were collected through the last prenatal health checkup and lifestyle questionnaires.

Abbreviations: ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; ASVs, amplicon sequence variants; BMI, body mass index; Sobs, observed species; CS, caesarean section; FMT, fecal microbiota transplantation; I, CS-born neonates with swabbing vaginal fluid intervention; NMDS, non-metric multidimensional scaling; OUT, operational taxonomic units; SA, spouse age; SCB, serum conjugated bilirubin; STB, serum total bilirubin; TSC, the transitional stool samples of caesarean-delivered newborns; TSN, the transitional stool samples of naturally delivered newborns; WHO, World Health Organization.

The protocol of our present research was approved by the Medical Ethics Committee of the Women and Children's Hospital, School of Medicine, Xiamen University (KY-2018-020). All procedures were conducted in accordance with the Declaration of Helsinki. All patients were required to provide written informed consent prior to participation. No adverse events were reported for any of the newborns in this study.

Treatment and sample collection

Pregnant women scheduled to have a CS surgery were administrated with prophylactic antibiotics 15 to 60 minutes before skin incision and divided into the two groups according to their willingness to have their newborns swabbed with the vaginal fluids. Before delivery (CS or natural delivery), a 7 × 5 cm four-layered piece of gauze was double-folded and soaked in sterile saline and then inserted into the lower vagina for at least 30 mins before the administration of prophylactic antibiotics, and then removed and kept at room temperature in a sterile collector. The gauze was then divided vertically into two equal parts. One part of the gauze was temporally stored in a 50 mL microcentrifuge tube for subsequent analyses of microbiome, while the other part was used to treat CS newborns (n = 16) as soon as they were born. The principle of 'center-to-periphery' was strictly followed during the swabbing procedure, which began on the lips, followed by the face, the thorax, the arms, the legs, and finally the back; the complete process took approximately 30 s for each newborn. Six vaginal-born and four CS-born babies were swabbed with gauze containing sterile saline solution and were used as positive or negative references. All treatments were performed in the delivery room. Transitional stool was first collected by sterile diapers between 36 h and 72 h after birth, and then 1-2 g of the sample was transferred into a 50 mL microcentrifuge tube. Approximately 1-2 g of fresh maternal faeces was collected into a 50 mL microcentrifuge tube at the last excretion before delivery. All samples were placed into containers of ice and transported to the laboratory within 1 h after collection and stored at -80°C for long-term storage until further processing.

Microbial DNA extraction and 16S rDNA amplification

Microbial DNA in all samples was extracted utilizing the MoBio Powersoil DNA Isolation Kit (QIAGEN, German) according to the manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) was used to determine the DNA concentration. The extracted DNA was stored at -20°C until further analysis. The hypervariable V3-V4 regions of the 16S rDNA gene were amplified with the specific primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTATCTAAT-3'). PCRs were performed in triplicate in a 50 μL mixture containing 5 μL of KOD buffer (10 ×), 5 μL of dNTPs (2 mM), 3 μL of $MgSO_4$ (25 mM), 1.5 μL of 341F/806R primer (10 μM), 1 μL of KOD Polymerase, and 100 ng of template DNA. The PCR reagents were purchased from TOYOBO, Japan, and the PCR protocol was carried out for 30 cycles using the following parameters: 94°C pre-denaturation for 2 min, 98°C denaturation for 10 s, 50°C annealing for 30 s, 68°C annealing for 30 s, and hold at 4°C. After amplification, all of the PCR products were pooled, purified, and quantified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) and the ABI Step-One-Plus Real-Time PCR System (Life Technologies, USA) according to the standard protocols.

16S rDNA sequencing and data processing

The microbiome profiles were analyzed by 16S rRNA gene amplicon sequencing with the Illumina MiSeq 250PE platform (Illumina, San Diego, CA, USA). USEARCH software (version 8.1.1861) was applied to turn paired-end sequencing reads into merged, denoised, chimera-free, inferred sample sequences, and the sequence processing steps are described below in detail. Raw sequencing reads were filtered using FASTP software (version 0.18.0) to remove adapters and low-quality reads that would affect the subsequent assembly and analysis to obtain clean high-quality paired-end reads. Paired-end reads were merged as raw tags using FLASH (version 1.2.11), with a minimum overlap of 10 bp and mismatch error rates of 20% (Magoč and Salzberg, 2011), and further merged as raw amplicon sequence variants (ASVs) with a minimum overlap of 10 bp. Noisy sequences of raw tags were filtered under specific filtering conditions to obtain clean tags. The high-quality clean tags were clustered into OTUs of ≥ 97% similarity using the UPARSE (version 9.2.64) pipeline. All chimeric tags were removed using the UCHIME algorithm, and effective tags were finally obtained for the next analysis step. The tag sequence with the highest abundance was selected as the representative sequence within each cluster. The representative OTU sequences were classified into organisms by a naive Bayesian model using the RDP classifier (version 2.2) (Wang et al., 2007) based on the SILVA database (version 132) (Pruesse et al., 2007).

Statistical and bioinformatic analyses

Because the number of subjects was less than 50, the Shapiro-Wilk test and Levene's test were performed to assess the normality of distributions and the homogeneity of variance, respectively. One-way analysis of variance (ANOVA) was performed to compare normally distributed continuous variables, while the Kruskal-Wallis H test was conducted to compare unevenly distributed variables. Comparisons of Alpha and Beta diversity between any two groups were performed by utilizing Welch's t test, whereas Kruskal-Wallis H test was used for the comparison among groups. Statistical

analysis of the clinical data was achieved by Statistic Package for Social Science software (SPSS) (SPSS Inc., USA) (version 26.0). $P < 0.05$ was considered to be statistically significant.

Alpha-diversity was assessed by the observed species (Sobs) index, inverse Simpson index, Shannon index, and Pielou evenness index, while beta-diversity was calculated by the Bray-Curtis distance and illustrated by NMDS analysis. Alpha- and beta-diversity, as well as the NMDS were generated in the R project Vegan package (version 2.5.3) (Dixon, 2003) and plotted in the R project ggplot2 package (version 2.2.1) (Wickham et al., 2016). Circular layout representations of species abundance were graphed using circos (version 0.69-3) (Krzywinski et al., 2009). Between groups, Venn analysis was performed in the R project VennDiagram package (version 1.6.16) (Chen and Boutros, 2011). A ternary plot of taxa abundance was plotted using the R ggtern package (version 3.1.0) (Hamilton and Ferry, 2018).

Results

Baseline clinical information of pregnant women

A total of 111 mother-newborn dyads were initially recruited. As shown in Figure S2, we excluded 85 pairs due to the lack of integrated bio-samples (n = 69), health check-up data (n = 9), and self-reported diseases information (n = 7). Finally, 26 mother-newborn dyads were ultimately included according to our inclusion and exclusion criteria, and they provided 78 samples for the subsequent analyses. Missing values of age (n = 2), enrolment age (n = 1), haemoglobin (n = 1), alanine aminotransferase (ALT) (n = 1), aspartate aminotransferase (AST) (n = 1), serum total bilirubin (STB) (n = 1), and serum conjugated bilirubin (SCB) (n = 1) were interpolated using the multiple imputation method. The basic information of the pregnant women is shown in Table 1. The median maternal age was 26.50 (25.00-27.25) years, and the average body mass index (BMI) was 20.82 ± 2.45. No participants in the present research subjected active or passive smoking. Other demographic and clinical indicators among groups were roughly similar except for spouse age (SA) and AST (ANOVA, P_{SA}=0.033; Kruskal-Wallis H test, P_{AST}=0.022). Despite a significant difference in AST, other maternal biological factors were well homogenized among the different groups. In combination with the clinical indices, participants in this study were overall of a relatively good health status, and the maternal clinical statuses were comparable.

Microbiome overview of the maternal and neonatal samples

The diversity of the microbiota in a given habitat reflects the composition and relative abundance of the community. Approximately 460 bp of PCR products were generated by amplifying the V3-V4 region of the 16S rRNA gene to compare the bacterial diversity among samples. DNA sequencing after quality filtering yielded 8.29 million paired-end reads, which further merged into 7.21 million tags, with a minimum of 64253 tags per sample (average of 92448 ± 8822), and ultimately formed 27801 operational taxonomic units (OTUs).

Rarefaction curves evaluated the OTU richness and represented whether a reasonable sampling size (sequencing depth) was used. As shown in Figure S3, the almost horizontal asymptotic curves indicated that the sequencing depth was sufficient for our research. The values of observed species (Sobs), Pielou evenness, Shannon, and inverse Simpson indices were calculated to thoroughly assess alpha-diversity. The Sobs index indicated the actual detected OTUs, while the Pielou evenness index referred to the species equitability within each group. The Simpson and Shannon indices measured the degree of species asynchrony and stability, and higher values represented higher richness, evenness, or both (Luo et al., 2017). As shown in Figure 1A, the highest Sobs value could be observed in the transitional **s**tool samples of **n**aturally delivered newborns (TSN), and the OTUs differed substantially between the transitional **s**tool samples of **c**aesarean-section newborns (TSC) and the TSN, which demonstrated that CS surgery reduced the actual number of species of newborn gut microbes compared with vaginal delivery. In contrast, the TSC group presented the lowest evenness according to the value of the Pielou evenness index (Figure 1B) and there was a significant difference between the TSC and TSN groups (Welch's t test, P_{Pielou}=0.001). The above analyses revealed that CS surgery led to an obvious alteration in bacterial richness and evenness. Good agreement was exhibited in the Simpson index and Shannon index, which suggested that the gut microbial composition of CS newborns was less diverse (Welch's t test, $P_{Simpson}$=0.017, $P_{Shannon}$<0.001), while a lesser degree of dominant bacteria and distribution homogeneity were observed in CS newborns with swabbing vaginal fluid **i**ntervention (I) (Figures 1C, D). The aggregate analyses of alpha-diversity indicated that the neonatal gut microbiota was affected by both the mode of delivery and the swabbing treatment. Beyond that, pregnant women who underwent different labour modes showed no significant differences in intestinal (Welch's t test, P_{Sobs}=0.169, P_{Pielou}=0.207, $P_{Simpson}$=0.081, $P_{Shannon}$=0.186) and vaginal fluid microbiota (Welch's t test, P_{Sobs}=0.152, P_{Pielou}=0.188, $P_{Simpson}$=0.246, $P_{Shannon}$=0.178).

We further investigated beta-diversity according to the Bray-Curtis distance to compare the microbial community structures among groups, as this method provided a model to describe the overall pattern of community composition based on OTUs. Interestingly, some results seem to be inconsistent with those obtained from alpha-diversity analyses (Figure 2A). Briefly, significant differences could be observed between the neonates of CS and I group (Adonis test, P= 0.001), as well as the CS and vaginally delivered newborns (Adonis test, P = 0.011). The gut microbiota of CS with vaginal seedings and vaginally delivered newborns seemed to show no substantial difference in terms of beta-diversity. The above-mentioned results hinted the microbial composition of neonates was highly dependent on the mode of delivery. Besides, the microbiome of

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TABLE 1 Maternal Clinical Information at Baseline.

Variables	Overall	VD	CS	I	*P* value
	N=26	n=6 (23.08%)	n=4 (15.38%)	n=16 (61.54%)	
Marriage Age (years)	26.50 (25.00-27.25)	26.00 (25.75-31.15)	25.25 ± 1.11	26.16 ± 0.70	0.707
Enrollment Age (years)	30.00 (27.00-32.00)	26.67 ± 1.33	33.25 ± 1.97	30.00 (28.55-32.00)	0.055
Spouse Age (years)	32.08 ± 5.52	26.98 ± 2.51	34.00 (34.00-40.00)	33.00 ± 1.11	0.033
Age of Menarche (years)	14.52 ± 1.68	14.00 ± 0.68	15.75 ± 0.75	14.41 ± 0.42	0.255
SBP (mmHg)	103.04 ± 9.91	105.17 ± 3.44	109.25 ± 5.31	100.69 ± 2.49	0.262
DBP (mmHg)	64.40 ± 7.62	65.33 ± 3.03	68.00 ± 4.97	63.15 ± 1.82	0.512
Height (cm)	158.82 ± 5.14	161.08 ± 2.27	160.50 ± 2.99	157.55 ± 1.18	0.288
Weight (Kg)	53.82 ± 7.91	53.35 ± 1.46	53.50 ± 4.94	54.08 ± 2.24	0.979
BMI (kg/m^2)	20.82 ± 2.45	20.03 ± 0.31	20.18 ± 1.28	21.27 ± 0.70	0.507
Hemoglobin (g/L)	122.60 ± 8.80	125.83 ± 3.04	114.00 (113.00-121.75)	122.98 ± 2.37	0.208
Leukocyte ($\times10^9$/L)	7.08 (6.50-8.01)	7.86 ± 0.75	5.95 (5.18-7.10)	7.29 ± 0.27	0.155
Platelet ($\times10^9$/L)	230.52 ± 41.64	232.50 ± 11.68	214.50 ± 4.13	233.78 ± 12.55	0.644
Blood Glucose (mmol/L)	4.82 ± 0.31	4.74 ± 1.60	4.79 ± 0.18	4.86 ± 0.07	0.720
ALT (U/L)	13.30 (10.75-18.02)	11.00 ± 1.02	39.52 ± 16.21	15.34 ± 1.33	0.120
AST (U/L)	16.90 (14.00-19.00)	14.18 ± 0.71	22.00 ± 2.86	17.34 ± 1.01	0.022
Albumin (g/L)	42.63 ± 5.78	44.03 ± 1.74	40.60 ± 1.64	41.55 (38.83-44.05)	0.350
STB (μmol/L)	10.82 ± 2.73	10.44 ± 0.97	7.10 (6.63-10.50)	11.64 ± 0.64	0.091
SCB (μmol/L)	3.59 ± 1.54	3.52 ± 0.52	2.91 ± 0.95	3.78 ± 0.40	0.613
SCr (μmol/L)	56.50 ± 11.18	58.05 ± 5.00	50.48 ± 3.80	57.43 ± 2.91	0.518
BUN (mmol/L)	3.07 ± 0.74	3.11 ± 0.24	2.87 ± 0.45	3.10 ± 0.20	0.856
Smoking (n, %)	0 (0)	0 (0)	0 (0)	0 (0)	–
Use of Antibiotic before Delivery (n, %)	0 (0)	0 (0)	0 (0)	0 (0)	–
Use of Prebiotics/Probiotics (n, %)	0 (0)	0 (0)	0 (0)	0 (0)	–

Shapiro-Wilk tests were performed to test the assumptions of normality. Normally distributed variables with even variance were presented as mean ± SD, skewed variables as median (lower quartile to upper quartile), and categorical variables as n (%). Continuous variables were compared using ANOVA or Kruskal-Wallis test depending on distribution.
VD, Pregnant women underwent vaginal delivery; CS, Pregnant women underwent cesarean delivery; I, Pregnant women underwent cesarean delivery with vaginal fluid swabbing intervention on their newborns; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; BMI, Body mass index; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; STB, Serum total bilirubin; SCB, Serum conjugated bilirubin; SCr, Serum creatinine; BUN, Blood Urea Nitrogen. No pregnant woman reported tobacco consumption, and the use of antibiotics as well as prebiotics or probiotics. Therefore, no statistical tests were performed. And the P values were displayed as '-'.

vaginal fluids and stools samples were different between pregnant women who underwent different labour modes (Adonis test, $P_{V\text{-}VN}$=0.034, $P_{F\text{-}FN}$=0.047). Further, the visualization of principal coordinate analysis (PcoA) and non-metric multidimensional scaling (NMDS) analysis based on the Bray-Curtis distance all displayed clear ordinations that indicate the gut microbes of the CS newborns with swabbing treatment were more similar with those of the vaginally delivered newborns, rather than CS babies (Figures 2B, C). The above-mentioned results concluded that the infant gut microbiota shared more features with maternal vaginal fluids, and the microbial composition of transitional stool samples in CS-born babies with vaginal seedings tended to be more similar to that of TSN samples. The detailed information of statistical analyses was shown in Table 2.

The variation in microbiota caused by delivery modes and treatment

The analysis at the genus level indicated the microbial community of CS newborns consisted primarily of *Bacteroides* (12.10%), *Lactobacillus* (6.38%), and *Escherichia-Shigella* (6.03%) after the vaginal seedings intervention (Table 3; Figures 3, 4), which was more closed to vaginally delivered babies. Among them, the

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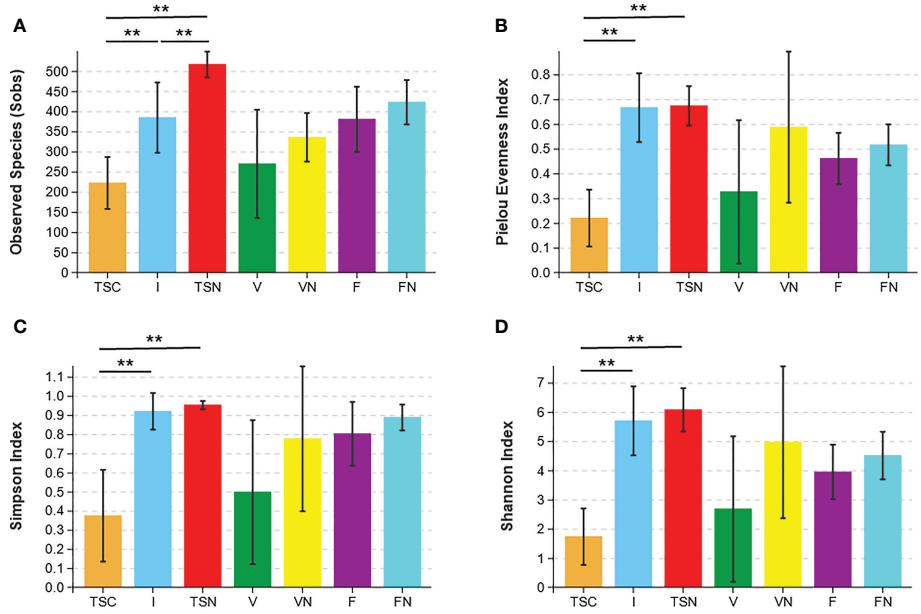

FIGURE 1
Microbial alpha diversity indices. The ecological diversity of microbiota in transitional stools of newborns (36-72h after birth), virginal fluids (before delivery) and stool of mothers (the last excretion before delivery) was measured by Sobs **(A)**, Pielou evenness index **(B)**, the Simpson index **(C)**, and the Shannon index **(D)**. The *P* values were conducted by Welch's t test. Statistical significance is displayed as **P*<0.05 and ***P*<0.01. Sobs, Observed species; TSC, Transitional stools of caesarean delivered neonates; I, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid; TSN, Transitional stools of natural delivered neonates; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; VN, Vaginal fluids of the pregnant women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery. Symbol "*" was presented above each plot.

genus of *Bacteroides* tended to be similar to those of vaginally delivered newborns (14.95%), which was significantly higher than CS-born neonates (0.15%). Statistical analysis further indicated that no obvious restoration pattern existed at the genus level, although the genus of *Faecalibacterium*, *Enterobacter*, and *Akkermansia* presented such trends (Table 3).

The VENN plots confirmed the above results from another aspect, which more shared taxa were detected between vaginally delivered and CS newborns in the intervention group, than those with CS newborns (Figure 5).

Discussion

In this population-based intervention study, the notion that neonatal microbial composition was dependent on the mode of delivery was confirmed by our results, which were in good agreement with previous researches (Fouhy et al., 2019). In addition, we observed the presence of restoration pattern of microbial composition at the genus level. It is a potential method of neonatal exposure to maternal vaginal microbiota shortly after CS birth to promote the development of the gut microbiome, which has gained traction in recent years (Obstetricians and Gynecologists, 2017). However, very few studies have focused on the microbiota in transitional stool to date. A pilot study conducted by Mueller et al. showed that significantly lower relative abundances of the genera *Bacteroidetes*, *Parabacteroides*, and *Clostridium* were observed in CS-born babies (Mueller et al., 2017b). In our present work, *Bacteroides* exhibited restoration pattern after vaginal seedings, while no other observed differences between the both CS groups. According to previous studies, the gut microbiome in CS-born infants might not be altered *via* vaginal seedings. Wayne S. Cutfield et al. assessed the effect of oral administration of maternal vaginal microbes to restore gut microbial composition among CS-born infants. However, there were no observed differences in gut microbiome composition between CS-born infants with or without the intervention at 1 month or 3 months after birth. And CS-born infants displayed the characteristic signature of low *Bacteroides* abundance compared with vaginal-born ones (Wilson et al., 2021). Our results may not align completely with others, which might be attributed to the dissimilarity in the collection, storage processing or analytic platforms used in the different studies (Perez-Muñoz et al., 2017). Maternal fecal microbiota transplantation (FMT) appears to be a more promising strategy. Willem M. de Vos et al. designed a proof-of-concept study that verified the gut microbial development could be restored rapidly *via* FMT (Korpela et al., 2020). Meanwhile, this study also demonstrated the neonatal gut microbiota is highly dependent on the delivery mode, which were in general agreement with our conclusions.

Interestingly, the microbiome appeared to show significant discrepancies in the vaginal fluid samples of pregnant women who

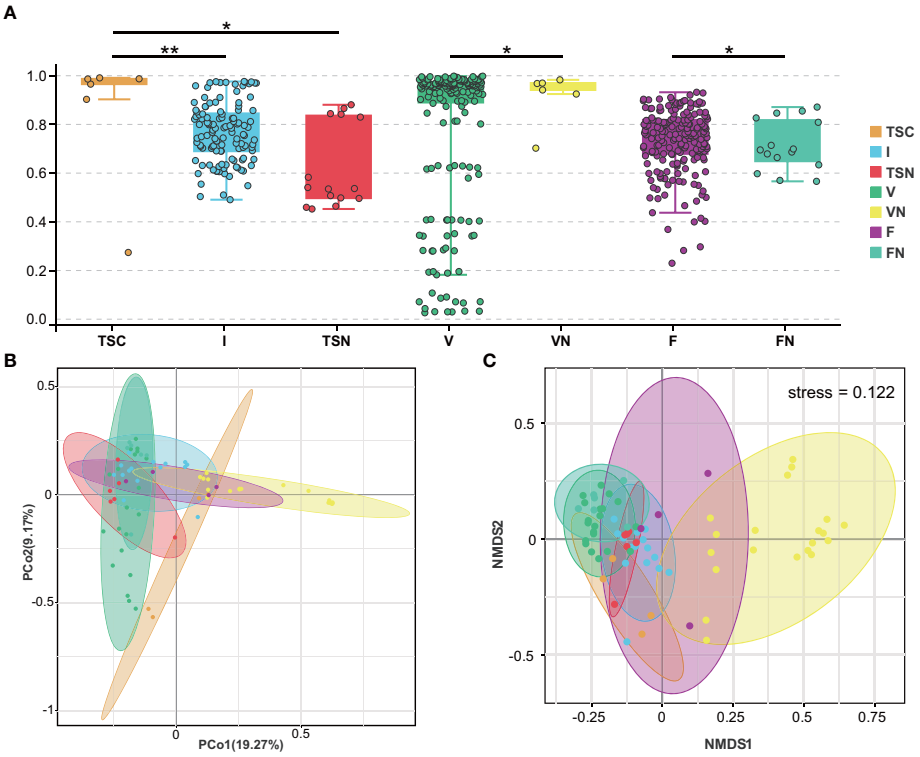

FIGURE 2
Visualization of beta-diversity index **(A)**, PcoA **(B)**, and NMDS **(C)**. These plots were conducted basing on Bray-Curtis Distance. Each dot represents one sample. In **(A)**, mean beta-diversity (distance from centroid) ± standard error.Statistical significance is displayed as *P < 0.05 and **P < 0.01. TSC, Transitional stools of caesarean delivered neonates; I, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid intervention; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

TABLE 2 Statistical analyses of beta-diversity.

Groups	*df*	F value	R^2	*P* value
F-vs-FN	1	1.784	0.069	0.056
V-vs-VN	1	2.207	0.091	0.043
F-vs-V	1	11.85	0.24	0.001
FN-vs-VN	1	2.11	0.21	0.004
TSC-vs-TSN	1	2.683	0.251	0.006
TSC-vs-I	1	2.814	0.135	0.004
TSN-vs-I	1	1.432	0.067	0.083
TSC-vs-F	1	2.692	0.109	0.007
I-vs-F	1	5.103	0.131	0.001
TSC-vs-V	1	3.151	0.125	0.001
I-vs-V	1	8.263	0.196	0.001
TSN-vs-FN	1	3.037	0.233	0.005
TSN-vs-VN	1	1.483	0.156	0.065

df, degree of freedom; TSC, Transitional stools of caesarean delivered neonates; I, Transitional stools of caesarean delivered neonates with the intervention of swabbing maternal vaginal fluids; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

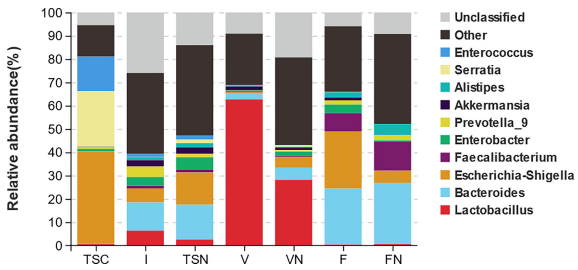

FIGURE 3
Taxa distribution plots at phylum and genus level. The relative abundances of microbial communities at genus level. TSC, Transitional stools of caesarean delivered neonates; I, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid intervention; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

were subjected to different labour modes. This conclusion was consistent with Marta Selma-Royo et al. (Selma-Royo et al., 2020) and Romero R et al. (Romero et al., 2014), which described the variations occurred during gestation, mainly in the intestinal and vaginal microbiomes. These studies also indicated that delivery mode significantly affected the maternal microbiota composition at delivery. One of the probable reasons was that medical decisions for different delivery modes depended on maternal health states and might be directly reflected in the vaginal microbiota. Moreover, previous investigations have proposed that the use of antibiotics during the gestation or the periparturient period would also cause those differences (Mueller et al., 2015b). Early studies observed that the abundance of the phylum Bacteroidetes participated in modulating the weight development of newborns (Ley et al., 2006; Turnbaugh et al., 2006). Additionally, it has been well documented that an increase in the abundance of the phylum *Proteobacteria* was associated with onset risks of diabetes and obesity (Su et al., 2018). In our present work, the increased richness of the phyla Bacteroidetes and the decreased richness of Proteobacteria might exert helpful impacts on the proper development of babies. At the genus level, the reduced abundance of pernicious bacteria *Escherichia-Shigella* was considered to be beneficial to CS neonates. Taken together, these findings indicate that the finer targeting of the vaginal fluids before exposure would determine the extent to which this intervention method could be popularized. The microbiome of transitional faeces reflects the colonization of microorganisms *in utero* as well as under the influence of external environmental factors shortly after birth (Mueller et al., 2017b). In this research, the gut bacterial composition of vaginally born babies was similar to that of the maternal vaginal fluid, but similar results could not be observed in CS newborns without treatment, while vertical transmission might be achieved by swabbing treatment among CS newborns. This phenomenon has aroused our concern. It is widely accepted that the effects of the natural delivery process are comprehensive, including physical, chemical, and biological effects, which generally last for one to two hours, covering the whole period of the second stage of labour (Sandall et al., 2018). During childbirth, newborns may inhale (swallow) the vaginal contents in their mother's birth canal (Butler É et al., 2020). The stomachs of newborns are pH neutral for several hours post birth as a result of swallowing the amniotic fluid *in utero*, thereby enabling the survival of ingested bacteria (Avery et al., 1966). However, the exposure application in our work deliberately avoided contact with the CS newborns' oral cavity, which might eliminate microbial colonization *via* the oral transmission route. Hence, the restoration by swabbing vaginal fluids may be inefficient due to incomplete biological effects and the absence

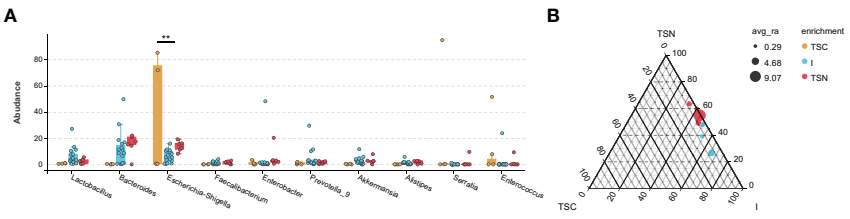

FIGURE 4
The Effect Analyses of Swabbing Exposure. The characteristic taxa were measured by indicator analysis **(A)** and ternary plot **(B)**. The P values were conducted by Tukey HSD test. Statistical significance is displayed as *P < 0.05 and **P < 0.01. TSC, Transitional stools of caesarean delivered neonates; I, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid intervention; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

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TABLE 3 Relative abundance at the genus level.

Relative abundance (%)	TSC	I	TSN	F	FN	V	VN
Lactobacillus	0.68	6.38	2.57	0.43	0.62	62.71	28.20
Bacteroides	0.15	12.10	14.95	23.95	26.26	2.77	5.12
Escherichia-Shigella	39.49	6.03	13.80	24.57	5.19	0.23	4.75
Faecalibacterium	0.10	1.19	1.34	7.88	12.59	0.24	0.45
Enterobacter	1.03	3.72	5.23	3.65	0.47	0.51	1.71
Prevotella_9	0.72	4.49	1.38	1.80	2.21	0.12	0.64
Akkermansia	0.25	2.72	2.87	1.26	0.10	1.67	1.14
Alistipes	0.00	1.19	1.77	2.23	4.69	0.59	0.37
Serratia	23.80	0.09	1.62	0.08	0.00	0.03	0.60
Enterococcus	14.81	1.50	1.63	0.02	0.00	0.02	0.03
Other	13.54	34.65	38.79	28.24	38.65	22.02	37.74
Unclassified	5.44	25.95	14.05	5.90	9.22	9.09	19.25

TSC, Transitional stools of caesarean delivered neonates; I, Transitional stools of caesarean delivered neonates with the intervention of swabbing maternal vaginal fluid; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

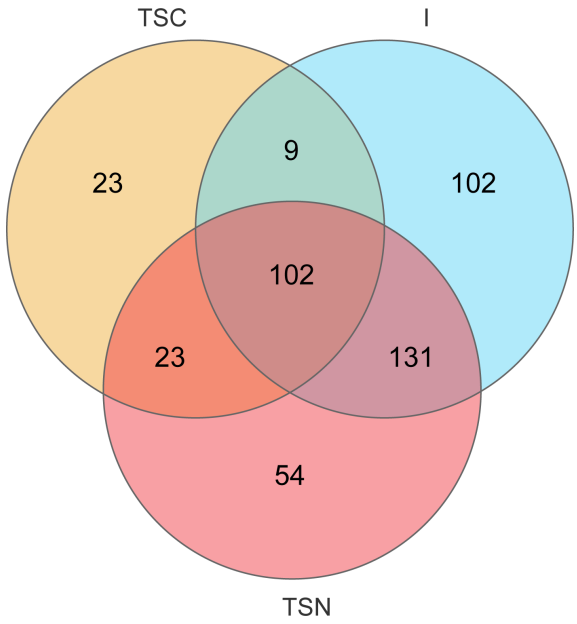

FIGURE 5
VENN diagrams at genus level. VENN diagram was used to represented the common and unique genera among different groups. TSC, Transitional stools of caesarean delivered neonates; I, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid; V, Vaginal fluids of the pregnant women who underwent caesarean delivery.

of chemical and physical effects. On the other hand, antibiotic therapy is a common preventive measure to avoid intra- or post-surgical infections. Nevertheless, residual concentrations of antibiotics in maternal blood might act on the newborn through circulation and last for a period of time post birth, which also means that the microorganisms in the vaginal secretions may not fully colonize the newborn because of the influence of the residual antibiotics.

Strengths and limitations

Strengths

The main strength is that our present study is the first research to our best knowledge focusing on the microbial transfer and the intervention programs for CS-born neonates using the samples of

transitional stools among Chinese population. The other strength is that the refined processes of sampling and experimental manipulations made the results more reliable and accurate.

Limitations

There were several main limitations in our research. First, due to the difficulty in sampling, the number of subjects in CS group was relatively small, potentially leading to misinterpretation. Second, this study was based on cross-sectional data, the dynamic changes of microbiome could not be observed, which limited the generalizability of this research.

Conclusion

Through the intervention project conducted in the Chinese population, we first verified the microbial alterations induced by different delivery modes, including noticeable changes in alpha- and beta-diversity, the structure of gastrointestinal bacterial communities. Second, the issue of the source of gut microbiota for newborns born *via* different delivery modes was partly resolved according to NMDS analysis. The structure of neonatal gut microbes shared more features with maternal vaginal samples among vaginally delivered babies. But such phenomenon could not be found among CS newborns without vaginal seedings. Last through ternary plots and Venn diagrams, we concluded that swabbing exposure could partly restored the dysbiosis of gut microbiota caused by CS.

Data availability statement

The data presented in the study are deposited in the NCBI repository (https://www.ncbi.nlm.nih.gov/sra), accession number PRJNA890171.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the Women and Children's Hospital, School of Medicine, Xiamen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CT and XZ contributed to design the study, research data and wrote the manuscript. JX and SH contributed to data interpretation and the discussion of the results. YTX contributed to methodology. JZ and YL contributed to design the study and critically reviewed the manuscript. YSX, QW, and LM contributed to subject recruitment, sample collection, and perform clinical examination. HS conceptualized and designed the protocol and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1065884/full#supplementary-material

References

Avery, G. B., Randolph, J. G., and Weaver, T. (1966). Gastric acidity in the first day of life. *Pediatrics* 37, 1005–1007. doi: 10.1542/peds.37.6.1005

Azad, M. B., Konya, T., Persaud, R. R., Guttman, D. S., Chari, R. S., Field, C. J., et al. (2016). Impact of maternal intrapartum antibiotics, method of birth and breastfeeding on gut microbiota during the first year of life: A prospective cohort study. *Bjog* 123, 983–993. doi: 10.1111/1471-0528.13601

Betran, A. P., Torloni, M. R., Zhang, J. J., and G Lmezoglu, A. M. (2016). WHO statement on caesarean section rates. *Bjog* 123, 667–670. doi: 10.1111/1471-0528.13526

Betran, A. P., Temmerman, M., Kingdon, C., Mohiddin, A., Opiyo, N., Torloni, M. R., et al. (2018). Interventions to reduce unnecessary caesarean sections in healthy women and babies. *Lancet* 392, 1358–1368. doi: 10.1016/S0140-6736(18)31927-5

Boerma, T., Ronsmans, C., Melesse, D. Y., Barros, A. J. D., Barros, F. C., Juan, L., et al. (2018). Global epidemiology of use of and disparities in caesarean sections. *Lancet* 392, 1341–1348. doi: 10.1016/S0140-6736(18)31928-7

Butler É, M., Chiavaroli, V., Derraik, J. G. B., Grigg, C. P., Wilson, B. C., Walker, N., et al. (2020). Maternal bacteria to correct abnormal gut microbiota in babies

born by c-section. *Med. (Baltimore)* 99, e21315. doi: 10.1097/MD.0000000000021315

Cegolon, L., Mastrangelo, G., Maso, G., Pozzo, G. D., Heymann, W. C., Ronfani, L., et al. (2020). Determinants of length of stay after cesarean sections in the friuli venezia giulia region (North-Eastern Italy), 2005-2015. *Sci. Rep.* 10, 19238. doi: 10.1038/s41598-020-74161-2

Chen, H., and Boutros, P. C. (2011). VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in r. *BMC Bioinf.* 12, 35. doi: 10.1186/1471-2105-12-35

Chu, D. M., Ma, J., Prince, A. L., Antony, K. M., Seferovic, M. D., and Aagaard, K. M. (2017). Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nat. Med.* 23, 314–326. doi: 10.1038/nm.4272

Dixon, P. (2003). VEGAN, a package of r functions for community ecology. *J. Vegetation Sci.* 14, 927–930. doi: 10.1111/j.1654-1103.2003.tb02228.x

Dominguez-Bello, M. G., Godoy-Vitorino, F., Knight, R., and Blaser, M. J. (2019). Role of the microbiome in human development. *Gut* 68, 1108–1114. doi: 10.1136/gutjnl-2018-317503

Ekstrom, L. D., Ahlqvist, V. H., Persson, M., Magnusson, C., and Berglind, D. (2020). The association between birth by cesarean section and adolescent cardiorespiratory fitness in a cohort of 339,451 Swedish males. *Sci. Rep.* 10, 18661. doi: 10.1038/s41598-020-75775-2

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., et al. (2018). Mother-to-Infant microbial transmission from different body sites shapes the developing infant gut microbiome. *Cell Host Microbe* 24, 133–145.e5. doi: 10.1016/j.chom.2018.06.005

Fouhy, F., Watkins, C., Hill, C. J., O'shea, C. A., Nagle, B., Dempsey, E. M., et al. (2019). Perinatal factors affect the gut microbiota up to four years after birth. *Nat. Commun.* 10, 1517. doi: 10.1038/s41467-019-09252-4

Hamilton, N. E., and Ferry, M. (2018). Ggtern: Ternary diagrams using ggplot2. *J. Stat. Software* 87, 1–17. doi: 10.18637/jss.v087.c03

He, Q., Kwok, L. Y., Xi, X., Zhong, Z., Ma, T., Xu, H., et al. (2020). The meconium microbiota shares more features with the amniotic fluid microbiota than the maternal fecal and vaginal microbiota. *Gut Microbes* 12, 1794266. doi: 10.1080/19490976.2020.1794266

Korpela, K., Helve, O., Kolho, K. L., Saisto, T., Skogberg, K., Dikareva, E., et al. (2020). Maternal fecal microbiota transplantation in cesarean-born infants rapidly restores normal gut microbial development: A proof-of-Concept study. *Cell* 183, 324–334.e5. doi: 10.1016/j.cell.2020.08.047

Korpela, K., Salonen, A., Veps L Inen, O., Suomalainen, M., Kolmeder, C., Varjosalo, M., et al. (2018). Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. *Microbiome* 6, 182. doi: 10.1186/s40168-018-0567-4

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., et al. (2009). Circos: An information aesthetic for comparative genomics. *Genome Res.* 19, 1639–1645. doi: 10.1101/gr.092759.109

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology: Human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Li, H. T., Luo, S., Trasande, L., Hellerstein, S., Kang, C., Li, J. X., et al. (2017). Geographic variations and temporal trends in cesarean delivery rates in china 2008-2014. *JAMA* 317, 69–76. doi: 10.1001/jama.2016.18663

Luo, M., Liu, Y., Wu, P., Luo, D. X., Sun, Q., Zheng, H., et al. (2017). Alternation of gut microbiota in patients with pulmonary tuberculosis. *Front. Physiol.* 8, 822. doi: 10.3389/fphys.2017.00822

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Martin, R., Makino, H., Cetinyurek Yavuz, A., Ben-Amor, K., Roelofs, M., Ishikawa, E., et al. (2016). Early-life events, including mode of delivery and type of feeding, siblings and gender, shape the developing gut microbiota. *PLoS One* 11, e0158498. doi: 10.1371/journal.pone.0158498

Mueller, N. T., Bakacs, E., Combellick, J., Grigoryan, Z., and Dominguez-Bello, M. G. (2015a). The infant microbiome development: Mom matters. *Trends Mol. Med.* 21, 109–117. doi: 10.1016/j.molmed.2014.12.002

Mueller, N. T., Mao, G., Bennet, W. L., Hourigan, S. K., Dominguez-Bello, M. G., Appel, L. J., et al. (2017a). Does vaginal delivery mitigate or strengthen the intergenerational association of overweight and obesity? findings from the Boston birth cohort. *Int. J. Obes. (Lond)* 41, 497–501. doi: 10.1038/ijo.2016.219

Mueller, N. T., Shin, H., Pizoni, A., Werlang, I. C., Matte, U., Goldani, M. Z., et al. (2017b). Delivery mode and the transition of pioneering gut-microbiota structure, composition and predicted metabolic function. *Genes (Basel)* 8. doi: 10.3390/genes8120364

Mueller, N. T., Whyatt, R., Hoepner, L., Oberfield, S., Dominguez-Bello, M. G., Widen, E. M., et al. (2015b). Prenatal exposure to antibiotics, cesarean section and risk of childhood obesity. *Int. J. Obes. (Lond)* 39, 665–670. doi: 10.1038/ijo.2014.180

Perez-Muñoz, M. E., Arrieta, M. C., Ramer-Tait, A. E., and Walter, J. (2017). A critical assessment of the "sterile womb" and "*in utero* colonization" hypotheses: Implications for research on the pioneer infant microbiome. *Microbiome* 5, 48. doi: 10.1186/s40168-017-0268-4

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., et al. (2007). SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188–7196. doi: 10.1093/nar/gkm864

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., et al. (2014). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2, 4.

Rusconi, F., Zugna, D., Annesi-Maesano, I., Ba Z, N., Barros, H., Correia, S., et al. (2017). Mode of delivery and asthma at school age in 9 European birth cohorts. *Am. J. Epidemiol.* 185, 465–473. doi: 10.1093/aje/kwx021

Sandall, J., Tribe, R. M., Avery, L., Mola, G., Visser, G. H., Homer, C. S., et al. (2018). Short-term and long-term effects of caesarean section on the health of women and children. *Lancet* 392, 1349–1357. doi: 10.1016/S0140-6736(18)31930-5

Seidu, A. A., Hagan, J. E.Jr., Agbemavi, W., Ahinkorah, B. O., Nartey, E. B., Budu, E., et al. (2020). Not just numbers: beyond counting caesarean deliveries to understanding their determinants in Ghana using a population based cross-sectional study. *BMC Pregnancy Childbirth* 20, 114. doi: 10.1186/s12884-020-2792-7

Selma-Royo, M., Garc A-Mantrana, I., Calatayud, M., Parra-Llorca, A., Mart Nez-Costa, C., and Collado, M. C. (2020). Maternal microbiota, cortisol concentration, and post-partum weight recovery are dependent on mode of delivery. *Nutrients* 12. doi: 10.3390/nu12061779

Stearns, J. C., Simioni, J., Gunn, E., Mcdonald, H., Holloway, A. C., Thabane, L., et al. (2017). Intrapartum antibiotics for GBS prophylaxis alter colonization patterns in the early infant gut microbiome of low risk infants. *Sci. Rep.* 7, 16527. doi: 10.1038/s41598-017-16606-9

Su, M., Nie, Y., Shao, R., Duan, S., Jiang, Y., Wang, M., et al. (2018). Diversified gut microbiota in newborns of mothers with gestational diabetes mellitus. *PLoS One* 13, e0205695. doi: 10.1371/journal.pone.0205695

The American College of Obstetricians and Gynecologists (2017). Committee opinion no. 725: Vaginal seeding. *Obstet Gynecol* 130, e274–e278. doi: 10.1097/AOG.0000000000002402

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Walter, J., and Hornef, M. W. (2021). A philosophical perspective on the prenatal *in utero* microbiome debate. *Microbiome* 9, 5. doi: 10.1186/s40168-020-00979-7

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wells, J. C., Wibaek, R., and Poullas, M. (2019). Global epidemiology of use of and disparities in caesarean sections. *Lancet* 394, 24–25. doi: 10.1016/S0140-6736(19)30715-9

Wickham, H., Chang, W., and Wickham, M. H. (2016). *Package 'ggplot2'. create elegant data visualisations using the grammar of graphics. version, 2.* 1–189.

Wilson, B. C., Butler É, M., Grigg, C. P., Derraik, J. G. B., Chiavaroli, V., Walker, N., et al. (2021). Oral administration of maternal vaginal microbes at birth to restore gut microbiome development in infants born by caesarean section: A pilot randomised placebo-controlled trial. *EBioMedicine* 69, 103443. doi: 10.1016/j.ebiom.2021.103443

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Xinyue Hu,
Nanchang University, China
Rongguo Li,
Jinan Maternity and Child Care Hospital, China
Yingjie Yang,
Guizhou Cancer Hospital, China
Silvia –Giono Cerezo,
Instituto Politécnico Nacional (IPN), Mexico

*CORRESPONDENCE
Bingbing Xiao
doctorxbb@163.com

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Association between human papillomavirus infection and common sexually transmitted infections, and the clinical significance of different *Mycoplasma* subtypes

Disi A, Hui Bi, Dai Zhang and Bingbing Xiao*

Department of Obstetrics and Gynecology, Peking University First Hospital, Beijing, China

Introduction: Human papillomavirus (HPV) infection, especially persistent high-risk HPV, is associated with cervical cancer. Female reproductive tract microecological disorders and lower genital tract infections have been increasingly correlated with HPV infection and cervical lesions. Due to their common risk factors and transmission routes, coinfection with other sexually transmitted infections (STIs) has become a concern. Additionally, the clinical significance of *Mycoplasma* subtypes appear to vary. This study aimed to assess the correlations between common STIs and HPV infection, and to investigate the clinical significance of *Mycoplasma* subtypes.

Methods: We recruited 1,175 patients undergoing cervical cancer screening at the Peking University First Hospital gynecological clinic from March 2021 to February 2022 for vaginitis and cervicitis tests. They all received HPV genotyping and detection of STIs, and 749 of them underwent colposcopy and cervical biopsy.

Results: Aerobic vaginitis/desquamative inflammatory vaginitis and STIs (mainly single STIs) were found significantly more often in the HPV-positive group than in the HPV-negative group. Among patients with a single STI, rates of infection with herpes simplex virus type 2 or UP6 in the HPV-positive group were significantly higher than in the HPV-negative group (OR_{adj}: 1.810, 95%CI: 1.211–2.705, P=0.004; OR_{adj}: 11.032, 95%CI: 1.465–83.056, P=0.020, respectively).

Discussion: Through detailed *Mycoplasma* typing, a correlation was found between different *Mycoplasma* subtypes and HPV infection. These findings suggest that greater attention should be paid to detecting vaginal microecological disorders in those who are HPV-positive. Further, lower genital tract infections, including both vaginal infections and cervical STIs, are significantly more common among women who are HPV-positive and who thus require more thorough testing. Detailed typing and targeted treatment of *Mycoplasma* should become more routine in clinical practice.

KEYWORDS

sexually transmitted infection, vaginal microecological disorder, human papillomavirus, cervical intraepithelial neoplasia, *Ureaplasma parvum*, herpes simplex virus type 2

Introduction

Human papillomavirus (HPV) is among the most common sexually transmitted infections (STIs), and persistent high-risk HPV (HR-HPV) infection is associated with cervical cancer (Tommasino, 2014). More than 200 HPV subtypes have been found to infect humans, among which 14 (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) have been identified as carcinogenic (Kombe Kombe et al., 2020; World Health Organization, 2021). More and more studies have shown that genital tract microecological disorder may be associated with HPV infection and cervical lesions (Zeng et al., 2022; Xu et al., 2023).

Genital tract infections, including vaginitis and cervicitis, can lead to genital tract microecological disorders. Common types of vaginitis, including bacterial vaginosis (BV), aerobic vaginitis (AV), desquamative inflammatory vaginitis (DIV), trichomoniasis (TV), vulvovaginal candidiasis (VVC), and cervicitis, are associated with STIs. STIs are a significant medical problem affecting women's health, with an estimated global daily incidence of 1 million (Unemo et al., 2017). Common causes of STIs include *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), herpes simplex virus type 2 (HSV-2), *Mycoplasma hominis* (MH), *Mycoplasma genitalium* (MG), *Ureaplasma urealyticum* (UU), and *Ureaplasma parvum* (UP). The World Health Organization estimates that CT infection, NG infection, syphilis, and TV together accounted for 357.4 million new infections globally in 2012 (Newman et al., 2015). The increasing prevalence of *Mycoplasma* infections and other STIs, including HSV-2 infection, and rates of antibiotic resistance are also concerning. Although earlier studies merely detected and analyzed "genital *Mycoplasma* infection" (Friedek et al., 2004; Zhang et al., 2010), it is now acknowledged that *Mycoplasma* can be further divided into subtypes with varying clinical significance, including MH, MG, UU, and UP. Among these, MH infection is found to associate with an increased risk of cervicitis, pelvic inflammatory disease, and infertility, whereas MG, UU, and UP can be positive in not only symptomatic but also frequently in healthy individuals (Zhang and Liu, 2016; Horner et al., 2018; Tuddenham et al., 2022).

In this study we recruited subjects undergoing cervical cancer screening and tested them for genital tract infection, including carrying out detailed typing of *Mycoplasma*, to investigate the correlation between common STIs and HPV infection. The aim of the study was to guide more efficient and comprehensive clinical examination and diagnosis.

Materials and methods

Study cohort and clinical sample collections

This human study was reviewed and approved by the Ethics Committee of Peking University First Hospital (2021KY062). We collected data on 1,175 subjects who underwent cervical cancer screening at gynecological clinics of The First Hospital of Peking University from March 2021 to February 2022 and who met the following requirements: age 19–50 years; pre-menopausal; sexual history; not in the menses phase of their menstrual cycle; and abstention from intercourse or vaginal medication or irrigation for 3 days before sample collection. Cervical samples were collected from all enrolled subjects for molecular detection of HPV and STI pathogens. Those referred for colposcopy underwent comprehensive colposcopy and pathological biopsy of any abnormal cervical tissue. Exclusion criteria were as follows: pregnancy within the previous 8 weeks; vaginal bleeding; history of genital tract tumors; recent treatment for HPV infection or other STI; a history of hysterectomy, cervical surgery, pelvic radiotherapy, or cervical ablation or resection in the last 12 months; and use of antibiotics or probiotics within the past month (Figure 1).

Samples were collected by professional gynecologists after standardized training. With the patient in the lithotomy position, vaginal secretions were collected with a swab at a standard anatomical site (one-third of the way up the lateral vaginal wall) and rolled onto a glass slide for immediate Gram staining to detect candidal hyphae and spores and clue cells. Exposing the cervix with a sterile speculum without lubricant, a sterile cotton swab was rotated five times in the cervical canal at a depth of 1–2 cm for 15–20 seconds before removal. Exfoliated cervical cells and secretions were obtained from the cervical epithelium with two cell brushes and stored at 4°C. They were then transferred to a buffer for DNA testing immediately or within 10 days, for separate genotyping of 21 HPV subtypes and STI pathogen detection.

BV was diagnosed by using the Gram stain-based Nugent score (0–3 was considered BV negative, 4–6 intermediate, and 7–10 BV positive) (Nugent et al., 1991) and, using the modified Amsel diagnostic criteria (Amsel et al., 1983) when three of the following were present: thin homogeneous discharge; a vaginal pH > 4.5; release of amines on addition of 10% potassium hydroxide to vaginal fluid; and the presence of clue cells. VVC was diagnosed by identification of budding yeasts, hyphae, or pseudo-hyphae in a wet preparation (saline, 10% potassium hydroxide) of vaginal discharge, or if Gram staining yielded a

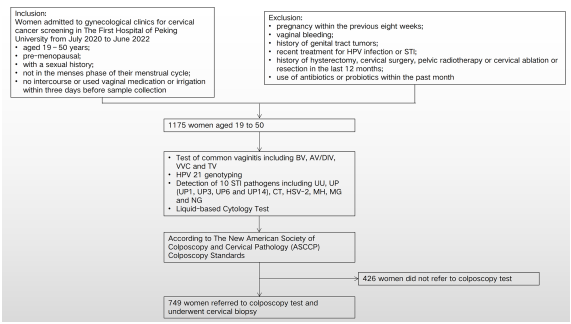

FIGURE 1
Flow chart and study design. HPV, human papillomavirus; STI, sexually transmitted infection; CT, *Chlamydia trachomatis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; UP, *Ureaplasma parvum*; NG, *Neisseria gonorrhoeae*; HSV-2, herpes simplex virus type 2; BV, bacterial vaginosis; AV/DIV, aerobic vaginitis/desquamative inflammatory vaginitis; TV, trichomoniasis vaginalis; VVC, vulvovaginal candidiasis.

positive result for a yeast species. TV was diagnosed by wet mount microscopy immediately following vaginal secretion swab. AV/DIV was diagnosed using the criteria proposed by Donders et al. (Donders et al., 2017), based on lactobacillary grade and the presence of other bacteria, leukocytes, and parabasal epithelial cells.

HPV genotyping and detection of sexually transmitted pathogens

The 21 HPV GenoArray Diagnostic Kit (HBGA-21PKG; HybriBio, Ltd., Chaozhou, China) was used, with the Rapid Capture System, using a HPV genotyping macroarray for HPV identification. The kit detects 14 HR-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 66), one suspected HR-HPV type (HPV53), and six low-risk HPV (LR-HPV) types (HPV6, 11, 42, 43, 44, and CP8304). We used the kit in accordance with the manufacturer's instructions and analyzed the results of cell lysis, DNA extraction, polymerase chain reaction (PCR) amplification, and hybridization.

A nucleic acid detection kit (HBRT-STD6; HybriBio, Ltd.) was used to detect STI pathogens including UU, UP (UP1, 3, 6, and 14), CT, HSV-2, MH, MG, and NG. A real-time PCR fluorescence probe was used to detect the pathogenic STI microorganisms. This method was used for single and mixed infections.

Liquid-based cytology test

Exfoliated cervical cell specimens were collected by one of two gynecologists using a conical cytobrush, placed into a tube containing 4 mL of preservation solution and temporarily stored at 4°C until testing. Cervical cytology samples were diagnosed using a ThinPrep® cytologic test (TCT) (TriPath Imaging, Inc., Burlington, VT, USA) in accordance with the manufacturer's instructions and double checked by cytotechnologists. Reported cytological results were classified in accordance with the 2001 Bethesda system (Solomon et al., 2002).

Colposcopy test

Colposcopy referral criteria were those of the most recent American Society of Colposcopy and Cervical Pathology (ASCCP) colposcopy standards (Wright, 2017), and colposcopy was performed following standard procedures. According to the ASCCP criteria and terminology, colposcopy impressions can be classified as benign, low-grade features, high-grade features, or cancer, as defined by the International Federation of Cervical Pathology and Colposcopy (Bornstein et al., 2012; Board, 2020; Höhn et al., 2021). In this study we categorized two groups for analyses: lower than low-grade squamous intraepithelial lesions (≤ LSIL), including benign and low-grade features, and higher than high-grade squamous intraepithelial lesions (≥ HSIL), including high-grade features and cancer.

Statistical analyses

IBM SPSS Statistics version 28.0 (IBM Corporation, Armonk, NY, USA) software was used for statistical analyses. Frequency data are described as a percentage of cases, and co-occurrence of HPV and other bacteria was compared using chi-squared and Fisher's exact probability tests, as appropriate. The Bonferroni correction test level was 0.05/3 = 0.0168 for multiple comparisons between different age groups. Univariate logistic regression was used to analyze the relative risk (odds ratio [OR] and 95% confidence interval [CI]) of HPV infection with different pathogens. After adjusting for age and AV/DIV infection status, the adjusted OR (OR_{adj}) and 95% CI were calculated. All tests were two-sided, and a p-value < 0.05 was considered statistically significant.

Results

Clinical characteristics and prevalence of HPV and STIs

A total of 1,175 subjects screened at Peking University First Hospital from March 2021 to February 2022 were enrolled. The age range of the study cohort was 19–50 years, with an average (± SD) age of 35.37 ± 6.773 years, and an approximately normal distribution. The overall HPV infection rate was 66.0% (775/1,175), with a monotypic infection rate of 54.2% (420/775) and a polytypic infection rate of 45.8% (355/775). The HR-HPV infection rate in the HPV-positive group was 95.7% (742/775), of which HPV16/18 infection accounted for 29.2% (217/742). Calculating the number of infections by HPV subtype revealed that the three most common HR-HPV subtypes were HPV16, HPV58, and HPV52, whereas the three most common low-grade HPV subtypes were HPV11, HPV CP8304, and HPV6 (Figure 2). For TCT, 97.4% (1,145/1,175) were ≤ LSIL, and 2.6% (30/1,175) were ≥ HSIL. A total of 63.7% of the cohort (749/1,175) underwent colposcopy and cervical biopsy in accordance with the most recent ASCCP colposcopy standards (Wright, 2017), among whom 82.9% (621/749) were ≤ LSIL and 17.1% (128/749) were ≥ HSIL.

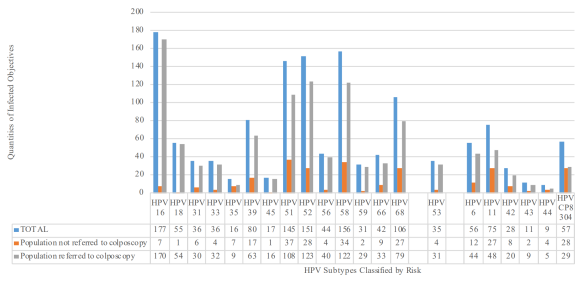

FIGURE 2
Number of people infected by different HPV subtypes classified by risk. HR-HPV subtypes include HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. Suspected HR-HPV subtypes include HPV53. LR-HPV subtypes include HPV6, 11, 42, 43, 44, and CP8304.

The overall STI pathogen rate was 61.0% (717/1,175), of which single and multiple infections accounted for 68.9% (494/717) and 31.1% (223/717), respectively. The most prevalent infections were UP3 infection, at 20.2% (237/1,175), followed by UP6 infection, at 18.3% (215/1,175), UU infection, at 14.3% (168/1,175), and CT infection, at 11.3% (133/1,175) (Figure 3). We also tested for common types of vaginitis, including BV, AV/DIV, TV, and VVC, which were detected in 8.1% (95/1,175), 10.8% (127/1,175), 0.1% (1/1,175), and 3.1% (36/1,175) of subjects, respectively.

Vaginal microbiome based on HPV infection status and TCT and biopsy results

There were no significant differences in BV, TV, or VVC infection rate between the groups with and without HPV infection; however, the AV/DIV infection rate was significantly higher in the HPV-positive group than in the HPV-negative group (p = 0.026, Supplementary Table 1). In the HPV-positive group, there were no significant differences in BV, AV/DIV, TV, or VVC infection rates between those with and those without HR-HPV infection (Supplementary Table 2). Likewise, in the HR-HPV-positive group, there were no significant differences in BV, AV/DIV, TV, or VVC infection rates between the groups with and without HPV16/18 (Supplementary Table 3). Moreover, when classified by single or multiple HPV infection type, there were no significant between-group differences in BV, AV/DIV, TV, or VVC infection rates (Supplementary Table 4). After adjusting for HPV infection status, there were no significant differences in BV, AV/DIV, TV, or VVC infection rate between groups based on TCT results (Supplementary Table 5). Those referred for colposcopy had a significantly higher AV/DIV infection rate than those not referred for colposcopy (p = 0.011, Supplementary Table 6), whereas there were no between-group differences in BV, AV/DIV, TV, or VVC infection rates between these groups (Supplementary Table 7).

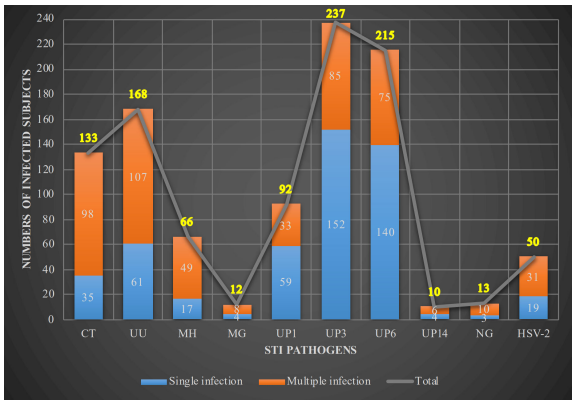

FIGURE 3
Types and proportions of the detected STI pathogens. Numbers of subjects with single infection, multiple infection, and total infection with detected STI pathogens shown. STI, sexually transmitted infection; CT, *Chlamydia trachomatis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; UP, *Ureaplasma parvum*; NG, *Neisseria gonorrhoeae*; HSV-2, herpes simplex virus type 2.

STIs in HPV infection status groups

Compared with the HPV-negative group, the overall STI pathogen rate, single STI pathogen rate, and multiple STI pathogens rate were all significantly higher (p < 0.001) in the HPV-positive group. Considering that there were > 50 potential STI pathogen combinations in the multiple STI group, and that that were < 15 cases of any combination, this group was combined for analyses. Although there was no age group effect for HPV infection rate (inconsistent with many previous studies), logistic regression was performed after adjusting for age and AV/DIV infection status. The UP6 (OR_{adj} 1.810, 95% CI 1.211–2.705; p = 0.004; Table 1) and HSV-2 (OR_{adj} 11.032, 95% CI 1.465–83.056; p = 0.020; Table 1) infection rates were significantly higher in the HPV-positive group than in the HPV-negative group. There were no other between-HPV infection group differences in STI pathogen rates.

As HR-HPV is a persistent infection associated with cervical cancer, we further analyzed the STI status in groups with or without HR-HPV infection in the HPV-positive group. The UP6 infection rate was significantly higher in the HR-HPV-negative group than in the HR-HPV-positive group (OR_{adj} 0.373, 95% CI 0.153–0.909; p = 0.030; Table 2), whereas there were no differences in the other STI pathogen rates between the HR-HPV infection status groups. Because the most commonly reported HR-HPV infection types are HPV16 and 18, the STI status in groups with or without HPV16/18 infections in the HR-HPV-positive group was also analyzed. There were no between- HPV16/18 infection group differences in any STI pathogen rate (Table 3).

Based on the number of HPV infection types, the STI status in the groups with various HPV infection genotypes was analyzed further, including single and multiple HPV infection profiles. There were no between-HPV infection genotype differences in STI pathogen rates (Table 4).

STIs in TCT status groups

Beyond the HPV–STI association, we explored whether or not STI may be associated with TCT results after adjustment for HPV infection status. Overall, there was not a between-TCT result group difference in STI pathogen rate after adjusting for HPV infection status. However, when analyzing only those with single infections, the STI infection rates for CT and MH were significantly higher in the group with TCT ≥ HSIL (p = 0.046 and p = 0.026, respectively; Table 5).

STIs in biopsy status groups

Because the relation between HPV infection and cervical intraepithelial neoplasia (CIN) is well known, we also assessed whether STI pathogen status differed between groups with diverse

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TABLE 1 Infection rates of STIs in groups with or without HPV infection.

		Single STI										Multiple STIs	STI negative
		CT	UU	MH	MG	UP1	UP3	UP6	UP14	NG	HSV-2		
n		35	61	17	4	59	152	140	4	3	19	223	458
HPV negative, *n* (%)		9 (2.5)	19 (4.8)	7 (1.8)	0 (0.0)	25 (6.3)	52 (13.0)	37 (9.3)	2 (0.5)	1 (0.3)	1 (0.3)	45 (11.3)	202 (50.5)
HPV positive, *n* (%)		26 (3.4)	42 (5.4)	10 (1.3)	4 (0.5)	34 (4.4)	100 (12.9)	103 (13.3)	2 (0.3)	2 (0.3)	18 (2.3)	178 (23.0)	256 (33.0)
Crude	OR	1.751	1.338	0.847	–	0.797	1.172	1.792	0.593	1.190	11.005	3.121	2.068
	95% CI	0.811–3.780	0.766–2.340	0.319–2.245	–	0.467–1.360	0.814–1.688	1.200–2.677	0.083–4.230	0.108–13.170	1.463–82.796	2.144–4.543	1.616–2.647
	p	0.154	0.307	0.738	0.999*	0.405	0.392	0.004	0.602	0.887	0.020	< 0.001	< 0.001
Adjusted	OR	1.637	1.289	0.672	–	0.803	1.194	1.810	0.627	1.138	11.032	3.072	2.069
	95% CI	0.754–3.554	0.735–2.260	0.243–1.854	–	0.470–1.371	0.829–1.721	1.211–2.705	0.088–4.477	0.102–12.699	1.465–83.056	2.104–4.487	1.617–2.648
	p	0.212	0.375	0.443	0.999*	0.422	0.341	0.004	0.642	0.917	0.020	< 0.001	< 0.001

Percentages are row percentages with respect to the corresponding group with different HPV infection status.
*Fisher's exact test.
Adjusted: Age, AV/DIV infection status.
STI, sexually transmitted infection; HPV, human papillomavirus; OR, odds ratio; CI, confidence interval; CT, *Chlamydia trachomatis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; UP, *Ureaplasma parvum*; NG, *Neisseria gonorrhoeae*; HSV-2, herpes simplex virus type 2.
-, Meaningless calculation results.

cervical biopsy results after adjusting for HPV infection status among the 749 individuals referred for colposcopy. There was not a significant between-cervical biopsy group difference in either total STI pathogen rate or individual STI pathogen rates after adjusting for HPV infection status (Table 6).

Discussion

Increasing attention has been paid in recent years to possible HPV risk factors, including lower genital tract infection and vaginal microecological disorder, to prevent cervical lesions (Fracella et al.,

TABLE 2 Infection rates of STIs in groups with or without HR-HPV infection among HPV-positive subjects.

		Single STI										Multiple STIs	STI negative
		CT	UU	MH	MG	UP1	UP3	UP6	UP14	NG	HSV-2		
n		26	42	10	4	34	100	103	2	2	18	178	256
HR-HPV negative, *n* (%)		2 (6.1)	2 (6.1)	0 (0.0)	0 (0.0)	0 (0.0)	4 (12.1)	8 (24.2)	0 (0.0)	0 (0.0)	0 (0.0)	10 (30.3)	7 (21.2)
HR-HPV positive, *n* (%)		24 (3.2)	40 (5.4)	10 (1.3)	4 (0.5)	34 (4.6)	96 (12.9)	95 (12.8)	2 (0.3)	2 (0.3)	18 (2.4)	168 (22.6)	249 (33.6)
Crude	OR	0.458	0.787	–	–	–	0.954	0.372	–	–	–	0.472	0.533
	95% CI	0.102–2.068	0.178–3.474	–	–	–	0.317–2.867	0.153–0.902	–	–	–	0.176–1.265	0.228–1.245
	p	0.310	0.751	0.999*	0.999*	0.998*	0.933	0.029	0.999*	0.999*	0.998*	0.136	0.146
Adjusted	OR	0.434	0.727	–	–	–	0.981	0.373	–	–	–	0.423	0.514
	95% CI	0.094–2.008	0.163–3.244	–	–	–	0.324–2.970	0.153–0.909	–	–	–	0.157–1.136	0.219–1.204
	p	0.285	0.676	0.999*	0.999*	0.998*	0.973	0.030	1.000*	1.000*	0.998*	0.088	0.125

Percentages are row percentages with respect to the corresponding group with different HPV infection status.
*Fisher's exact test.
Adjusted: Age, AV/DIV infection status.
STI, sexually transmitted infection; HPV, human papillomavirus; OR, odds ratio; CI, confidence interval; CT, *Chlamydia trachomatis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; UP, *Ureaplasma parvum*; NG, *Neisseria gonorrhoeae*; HSV-2, herpes simplex virus type 2.
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TABLE 3 Infection rates of STIs in groups with or without HPV 16/18 infection among HR-HPV-positive subjects.

		Single STI										Multiple STIs	STI negative
		CT	UU	MH	MG	UP1	UP3	UP6	UP14	NG	HSV-2		
n		24	40	10	4	34	96	95	2	2	18	168	249
HPV 16/18 negative, *n* (%)		13 (2.5)	32 (6.1)	5 (1.0)	4 (0.8)	24 (4.6)	61 (11.6)	66 (12.6)	2 (0.4)	0 (0.0)	14 (2.7)	126 (24.0)	178 (33.9)
HPV 16/18 positive, *n* (%)		11 (5.1)	8 (3.7)	5 (2.3)	0 (0.0)	10 (4.6)	35 (16.1)	29 (13.4)	0 (0.0)	2 (0.9)	4 (1.8)	42 (19.4)	71 (32.7)
Crude	OR	1.992	0.549	2.318	–	0.947	1.385	1.002	–	–	0.643	0.836	1.055
	95% CI	0.874–4.538	0.248–1.218	0.662–8.110	–	0.443–2.025	0.875–2.194	0.621–1.616	–	–	0.209–1.983	0.536–1.304	0.754–1.476
	p	0.101	0.140	0.188	0.999*	0.888	0.165	0.993	0.999*	0.999*	0.442	0.429	0.756
Adjusted	OR	1.921	0.535	2.427	–	0.951	1.396	1.004	–	–	0.649	1.366	1.034
	95% CI	0.834–4.425	0.241–1.189	0.657–8.967	–	0.444–2.035	0.880–2.215	0.622–1.621	–	–	0.211–2.002	0.726–2.569	0.737–1.450
	p-value	0.125	0.125	0.184	0.999*	0.896	0.156	0.987	0.999*	0.999*	0.452	0.334	0.847

Percentages are row percentages with respect to the corresponding group with different HPV infection status.
*Fisher's exact test.
Adjusted: Age, AV/DIV infection status.
STI, sexually transmitted infection; HPV, human papillomavirus; OR, odds ratio; CI, confidence interval; CT, *Chlamydia trachomatis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; UP, *Ureaplasma parvum*; NG, *Neisseria gonorrhoeae*; HSV-2, herpes simplex virus type 2.
–, Meaningless calculation results.

2022; Guo et al., 2022). In this study we found that AV/DIV infection was higher in the HPV-positive group than in the HPV-negative group ($p = 0.026$), although no significant between-group differences in BV, TV, or VVC infection rates were observed. Previous investigators have shown a correlation between common vaginitis and HPV infection, suggesting that this is a risk factor (Wu et al., 2022; Huang et al., 2023). BV is an infectious disease caused by the displacement of vaginal *Lactobacillus* by potentially

TABLE 4 Infection rates of STIs in groups with monotypic and multiple-type infection of HPV among HPV-positive subjects.

		Single STI										Multiple STIs	STI negative
		CT	UU	MH	MG	UP1	UP3	UP6	UP14	NG	HSV-2		
n		26	42	10	4	34	100	103	2	2	18	178	256
HPV monotypic infection, *n* (%)		12 (2.9)	30 (7.1)	5 (1.2)	2 (0.5)	19 (4.5)	64 (15.2)	57 (13.6)	2 (0.5)	0 (0.0)	0 (0.0)	71 (16.9)	158 (37.6)
HPV polytypic infection, *n* (%)		14 (3.9)	12 (3.4)	5 (1.4)	2 (0.6)	15 (4.2)	36(10.1)	46 (13.0)	0 (0.0)	2 (0.6)	18 (5.1)	107 (30.1)	98 (27.6)
Crude	OR	1.680	0.541	1.416	1.411	1.118	0.756	1.167	–	–	–	2.430	1.581
	95% CI	0.763–3.698	0.271–1.078	0.405–4.943	0.197–10.082	0.557–2.246	0.484–1.180	0.760–1.790	–	–	–	1.642–3.595	1.166–2.146
	p	0.197	0.081	0.586	0.732	0.754	0.219	0.480	0.999*	0.999*	0.998*	< 0.001	0.003
Adjusted	OR	1.761	0.540	1.750	1.327	1.123	0.747	1.161	–	–	–	2.413	1.596
	95% CI	0.794–3.906	0.270–1.079	0.476–6.431	0.184–9.543	0.559–2.258	0.478–1.168	0.756–1.782	–	–	–	1.626–3.583	1.175–2.168
	p	0.164	0.081	0.399	0.779	0.744	0.201	0.493	0.999*	0.999*	0.998*	< 0.001	0.003

Percentages are row percentages with respect to the corresponding group with different HPV infection status.
*Fisher's exact test.
Adjusted: Age, AV/DIV infection status.
STI, sexually transmitted infection; HPV, human papillomavirus; OR, odds ratio; CI, confidence interval; CT, *Chlamydia trachomatis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; UP, *Ureaplasma parvum*; NG, *Neisseria gonorrhoeae*; HSV-2, herpes simplex virus type 2.
–, Meaningless calculation results.

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TABLE 5 Infection rates of STIs in groups with different TCT results.

		Single STI										Multiple STIs	STI negative
		CT	UU	MH	MG	UP1	UP3	UP6	UP14	NG	HSV-2		
n		35	61	17	4	59	152	140	4	3	19	223	458
TCT ≤ LSIL, *n* (%)		32 (2.8)	59 (5.2)	15 (1.3)	4 (0.3)	59 (5.2)	148 (12.9)	137 (12.0)	4 (0.3)	3 (0.3)	19 (1.7)	217 (19.0)	448 (39.1)
TCT ≥ HSIL, *n* (%)		3 (10.0)	2 (6.7)	2 (6.7)	0 (0.0)	0 (0.0)	4 (13.3)	3 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (20.0)	10 (33.3)
Crude	OR	4.000	1.339	5.533	–	–	1.054	0.825	–	–	–	1.239	1.286
	95% CI	1.135–14.102	0.307–5.831	1.192–25.677	–	–	0.355–3.128	0.243–2.803	–	–	–	0.444–3.452	0.596–2.772
	p	0.031	0.697	0.029	0.999*	0.997*	0.924	0.758	0.999*	0.999*	0.998*	0.682	0.522
Adjusted	OR	3.643	1.265	5.867	–	–	1.020	0.736	–	–	–	0.971	1.099
	95% CI	1.026–12.942	0.289–5.533	1.241–27.745	–	–	0.343–3.036	0.216–2.513	–	–	–	0.343–2.746	0.506–2.388
	p	0.046	0.755	0.026	0.999*	0.997*	0.971	0.625	0.999*	0.999*	0.998*	0.955	0.812

Percentages are row percentages with respect to the corresponding group with different TCT results.
*Fisher's exact test.
Adjusted: HPV infection.
TCT, liquid-based cytology test; STI, sexually transmitted infection; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; OR, odds ratio; CI, confidence interval; CT, *Chlamydia trachomatis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; UP, *Ureaplasma parvum*; NG, *Neisseria gonorrhoeae*; HSV-2, herpes simplex virus type 2.
–, Meaningless calculation results.

pathogenic microorganisms such as *Gardnerella vaginalis* and *Prevotella* species, resulting in vaginal microecology imbalance. A possible correlation between BV and HPV infection and cervical lesions has also been reported (Usyk et al., 2022; Martins et al., 2023). However, the relationship between TV, VVC, and HPV infection remain unclear (Wang et al., 2020; Heikal et al., 2023). AV/DIV, identified relatively late compared with other types of vaginitis, is characterized by *Lactobacillus* decline and increases in

TABLE 6 Infection rates of STIs in groups with different cervical biopsy results.

		Single STI										Multiple STIs	STI negative
		CT	UU	MH	MG	UP1	UP3	UP6	UP14	NG	HSV-2		
n		27	41	13	3	40	94	96	3	2	14	144	272
≤ LSIL, *n* (%)		26 (4.2)	32 (5.2)	10 (1.6)	3 (0.5)	35 (5.6)	82 (13.2)	76 (12.2)	2 (0.3)	2 (0.3)	13 (2.1)	115 (18.5)	225 (36.2)
≥ HSIL, *n* (%)		1 (0.8)	9 (7.0)	3 (2.3)	0 (0.0)	5 (3.9)	12 (9.4)	20 (15.6)	1 (0.8)	0 (0.0)	1 (0.8)	29 (22.7)	47 (36.7)
Crude	OR	0.188	1.481	1.550	–	0.716	0.713	1.432	2.571	–	0.387	1.207	0.979
	95% CI	0.025–1.405	0.684–3.209	0.419–5.736	–	0.273–1.875	0.373–1.364	0.828–2.477	0.231–28.636	–	0.050–2.992	0.722–2.019	0.660–1.453
	p	0.103	0.319	0.512	0.999*	0.496	0.307	0.199	0.442	0.999*	0.327	0.473	0.917
Adjusted	OR	0.177	1.535	1.743	–	0.831	0.649	1.315	3.546	–	–	1.095	0.898
	95% CI	0.024–1.324	0.699–3.371	0.454–6.685	–	0.312–2.212	0.338–1.247	0.756–2.286	0.272–46.308	–	0.042–2.531	0.650–1.845	0.601–1.341
	p	0.092	0.286	0.418	0.999*	0.710	0.194	0.332	0.334	0.999*	0.284	0.733	0.600

Percentages are row percentages with respect to the corresponding group with different biopsy results.
*Fisher's exact test.
Adjusted: HPV infection.
STI, sexually transmitted infection; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; OR, odds ratio; CI, confidence interval; CT, *Chlamydia trachomatis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; UP, *Ureaplasma parvum*; NG, *Neisseria gonorrhoeae*; HSV-2, herpes simplex virus type 2.
–, Meaningless calculation results.

multiple aerobes, including *Streptococcus agalactiae* and *Streptococcus anginosus* (Donders et al., 2002). Previous studies have found that AV/DIV may be correlated with adverse pregnancy outcomes, such as abortion, stillbirth, premature delivery, and premature rupture of membranes, possibly due to toxin production or local effects on immunity, which in turn lead to infection (Han et al., 2019; Ma et al., 2022; Ncib et al., 2022).

Limited studies have previously been conducted to assess the association between AV/DIV and HPV infection. Jahic et al. (Jahic et al., 2013) found that AV/DIV may be associated with cervical LSIL. Vieira-Baptista et al. (Vieira-Baptista et al., 2016) found that moderate or severe AV/DIV, rather than BV, was independently associated with increased risk for major cervical cytological abnormalities. In addition, Plisko et al. (Plisko et al., 2021) found that moderate to severe AV/DIV and smoking were the most significant factors contributing to the development of CIN in HPV-positive women, especially high-grade CIN. Current speculation regarding pathogenesis includes that there is a link between AV/DIV, characterized by various degrees of inflammation and present with increased vaginal leucocytes, and highly increased concentrations of interleukin 1 beta (IL-1β) and IL-6, which are also characteristic of progressive CIN (Donders et al., 2017). Although additional large-sample studies will be needed to confirm and clarify this association, we suggest that greater attention is paid to AV/DIV in the association between vaginal microbiome and cervical lesions.

Recent studies have also identified a possible association between STIs and HPV infection. For instance, several studies have found that CT infection is associated with a high risk of, and persistent infection with, HPV (Naldini et al., 2019; Chen et al., 2020). Studies of the association between NG and HPV infection have been few. Others have found that persistent MH infection is associated with a high risk of HPV infection and CIN (Alotaibi et al., 2020; Klein et al., 2020). Similar correlations have been shown between UU and HPV infection (Kim et al., 2018). In this study, both single and multiple STI pathogen rates were found to be significantly higher in the HPV-positive group than in the HPV-negative group. The difference was particularly marked in the case of UP6 and HSV-2 infection rates (which refers to infection with only one of these organisms). However, by contrast, the UP6 infection rate was significantly higher in the HR-HPV-negative group than in the HR-HPV-positive group.

Detailed *Mycoplasma* typing has received insufficient clinical attention, partly because specific detection methods were lacking. In this study, we performed detailed *Mycoplasma* typing and, after analyzing the associations between common STI pathogens and HPV infection, were surprised to find significant differences in several UP subtype infection rates based on HPV infection status. To date, 14 *Ureaplasma* serovars have been identified, divided into two species: UP contains serovars 1, 3, 6, and 14, and UU contains the remaining 10 (Xiao et al., 2011). Few studies have addressed the association between UP and HPV. In 2016, Drago et al. (Drago et al., 2016) proposed that UP is a possible HPV-induced CIN enhancer agent, and confirmed this in a study 5 years later, showing that UP infection is a risk factor for persistent genital HPV infection (Ciccarese et al., 2021). In their study of 283 patients, Noma et al. (Noma et al., 2021) found that UP and HR-HPV coinfection increased LSIL risk. Similarly, a study of 480 patients found that UP14 and HR-HPV coinfection increased HSIL and cervical cancer risks, as did UP1 coinfection (Wang et al., 2019). A retrospective study of 668 patients found that UP6 infection was a risk factor for both HR-HPV infection and CIN, and that UP3 infection was a risk factor for CIN (Xie et al., 2021). Moreover, the possible role of UP in other illnesses has been investigated. Zanotta et al. found active UP3 infection in women with asymptomatic HR-HPV, and with idiopathic infertility, thus presenting a possible therapy target (Zanotta et al., 2019). Analogously, Rittenschober-Böhm et al. (Rittenschober-Böhm et al., 2019) observed that UP3 colonization increased the risk of spontaneous preterm birth and may be a target for therapeutic intervention studies. In this study, we found a significantly higher UP6 infection rate in the HPV-positive group than in the HPV-negative group (OR_{adj} 1.810, 95% CI 1.211–2.705; $p = 0.004$). Furthermore, the UP6 infection rate was significantly higher in the HR-HPV-negative group than in the HR-HPV-positive group (OR_{adj} 0.373, 95% CI 0.153–0.909; $p = 0.030$), suggesting that UP6 infection may be a risk factor for HPV infection. Our results are not uniformly consistent with those of previous studies, probably because of cohort differences or sample size limitations. Although the correlation between different UP serovars and HPV infection remains to be clearly established, based on our extant findings we nevertheless propose that further, detailed classification of *Mycoplasma*, especially different UP serovars, is needed to advance clinical practice.

Furthermore, the HPV infection rate group was significantly higher in the HSV-2-positive than in the HSV-2-negative group (OR_{adj} 11.032, 95% CI 1.465–83.056; $p = 0.020$). HSV-2 infection is among the most common STIs worldwide and is the leading cause of recurrent genital herpes. Many previous studies have used blood samples to detect HSV-2, partly because a specific kit for testing cervical samples was unavailable at the time. In this study, we used cervical samples to detect HSV-. Regarding the correlation between HSV-2 and HPV infection and cervical lesions, Smith et al. (Smith et al., 2002) found that HSV-2 seropositivity was associated with increased risks of squamous cell carcinoma (OR 2.19, 95% CI 1.41–3.40) and adeno- and adenosquamous cell carcinomas (OR 3.37, 95% CI 1.47–7.74), after adjustment for potential confounders. A cross-sectional study in 2020 observed significant differences in HSV-2 seroprevalence and HSV-2 active infection rates between negative and positive HR-HPV cases (Bahena-Román et al., 2020). Li et al. (Li and Wen, 2017) used eight datasets and a sample of 8,184 participants, finding that HSV-2 was associated with cervical cancer after adjusting for HR-HPV (OR_{adj} 1.90, 95% CI 1.09–3.34), suggesting that HSV-2 serostatus may serve as an independent predictor of cervical cancer. Our results appear to be consistent with these studies, though they are limited by the small sample size and the potential correlation between HSV-2 and HPV infection requires further study.

The main study limitation was its cross-sectional observational design. The sample size was also relatively small. Thus, larger, prospective studies are needed to confirm the relationship between STIs and HPV infection. Additional basic research is also needed to explain the specific underlying mechanisms.

In conclusion, HPV infection is associated with a disordered lower genital tract microecology, with a total STI pathogen rate of 61.0% in this cohort of participants who were undergoing routine cervical cancer screening. The total STI rate and multiple STI rate, as well as AV/DIV infection rate in HPV-positive patients were significantly higher than those in HPV-negative patients, which suggests that attention should be paid to the screening of lower genital tract infection in cervical cancer screening women, what's more, especially the STI screening in HPV positive patients. Specifically, HSV-2 and *Mycoplasma*, especially UP6, infection rates were significantly higher in the HPV-positive group, emphasizing the importance of detailed typing and targeted treatment of *Mycoplasma* in clinical practice. Prospective cohort studies are now needed to further explore the relations and mechanisms between STI pathogens and HPV infection, and persistent infection and cervical lesions, to improve cervical cancer prevention, screening, diagnosis, and treatment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee of Peking University First Hospital (2021KY062). The patients/participants provided their written informed consent to participate in this study.

Author contributions

BX conceived the study design. BX, DZ, and HB recruited volunteers and collected samples. DA and BX performed the data analysis. DA wrote the initial manuscript. BX and DA revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1145215/full#supplementary-material

References

Alotaibi, H. J., Almajhdi, F. N., Alsaleh, A. N., Obeid, D. A., Khayat, H. H., Al-Muammer, T. A., et al. (2020). Association of sexually transmitted infections and human papillomavirus co-infection with abnormal cervical cytology among women in Saudi Arabia. *Saudi J. Biol. Sci.* 27 (6), 1587–1595. doi: 10.1016/j.sjbs.2020.03.021

Amsel, R., Totten, P. A., Spiegel, C. A., Chen, K. C., Eschenbach, D., and Holmes, K. K. (1983). Nonspecific vaginitis. diagnostic criteria and microbial and epidemiologic associations. *Am. J. Med.* 74 (1), 14–22. doi: 10.1016/0002-9343(83)91112-9

Bahena-Román, M., Sánchez-Alemán, M. A., Contreras-Ochoa, C. O., Lagunas-Martínez, A., Olamendi-Portugal, M., López-Estrada, G., et al. (2020). Prevalence of active infection by herpes simplex virus type 2 in patients with high-risk human papillomavirus infection: A cross-sectional study. *J. Med. Virol.* 92 (8), 1246–1252. doi: 10.1002/jmv.25668

Board, W. (2020). *WHO classification of tumours: female genital tumours* (Lyon (France: IARC Publications), 1–632.

Bornstein, J., Bentley, J., Bösze, P., Girardi, F., Haefner, H., Menton, M., et al. (2012). 2011 colposcopic terminology of the international federation for cervical pathology and colposcopy. *Obstet Gynecol* 120 (1), 166–172. doi: 10.1097/AOG.0b013e318254f90c

Chen, H., Luo, L., Wen, Y., He, B., Ling, H., Shui, J., et al. (2020). *Chlamydia trachomatis* and human papillomavirus infection in women from southern hunan province in China: A Large observational study. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.00827

Ciccarese, G., Herzum, A., Pastorino, A., Dezzana, M., Casazza, S., Mavilia, M. G., et al. (2021). Prevalence of genital HPV infection in STI and healthy populations and risk factors for viral persistence. *Eur. J. Clin. Microbiol. Infect. Dis.* 40 (4), 885–888. doi: 10.1007/s10096-020-04073-6

Donders, G. G. G., Bellen, G., Grinceviciene, S., Ruban, K., and Vieira-Baptista, P. (2017). Aerobic vaginitis: no longer a stranger. *Res. Microbiol.* 168 (9-10), 845–858. doi: 10.1016/j.resmic.2017.04.004

Donders, G. G., Vereecken, A., Bosmans, E., Dekeersmaecker, A., Salembier, G., and Spitz, B. (2002). Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis. *Bjog* 109 (1), 34–43. doi: 10.1111/j.1471-0528.2002.00432.x

Drago, F., Herzum, A., Ciccarese, G., Dezzana, M., Casazza, S., Pastorino, A., et al. (2016). *Ureaplasma parvum* as a possible enhancer agent of HPV-induced cervical intraepithelial neoplasia: Preliminary results. *J. Med. Virol.* 88 (12), 2023–2024. doi: 10.1002/jmv.24583

Fracella, M., Oliveto, G., Sorrentino, L., Roberto, P., Cinti, L., Viscido, A., et al. (2022). Common microbial genital infections and their impact on the innate immune response to HPV in cervical cells. *Pathogens* 11 (11):1361. doi: 10.3390/pathogens11111361

Friedek, D., Ekiel, A., Chełmicki, Z., and Romanik, M. (2004). [HPV, *Chlamydia trachomatis* and genital *Mycoplasma*s infections in women with low-grade squamous intraepithelial lesions (LSIL)]. *Ginekol Pol.* 75 (6), 457–463.

Guo, C., Dai, W., Zhou, Q., Gui, L., Cai, H., Wu, D., et al. (2022). Cervicovaginal microbiota significantly changed for HPV-positive women with high-grade squamous intraepithelial lesion. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.973875

Han, C., Li, H., Han, L., Wang, C., Yan, Y., Qi, W., et al. (2019). Aerobic vaginitis in late pregnancy and outcomes of pregnancy. *Eur. J. Clin. Microbiol. Infect. Dis.* 38 (2), 233–239. doi: 10.1007/s10096-018-3416-2

Heikal, E. A., Elamir, A. M., Hegazi, M. A., Salem, H. S., Tawfeik, A. M., Bosilah, A. H., et al. (2023). Signature of real-time PCR in detection of trichomonas vaginalis infection and its association with human papillomavirus genotype 16. *Eur. Rev. Med. Pharmacol. Sci.* 27 (2), 501–510. doi: 10.26355/eurrev_202301_31050

Höhn, A. K., Brambs, C. E., Hiller, G. G. R., May, D., Schmoeckel, E., and Horn, L. C. (2021). 2020 WHO classification of female genital tumors. *Geburtshilfe Frauenheilkd* 81 (10), 1145–1153. doi: 10.1055/a-1545-4279

Horner, P., Donders, G., Cusini, M., Gomberg, M., Jensen, J. S., and Unemo, M. (2018). Should we be testing for urogenital *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* in men and women? - a position statement from the European STI guidelines editorial board. *J. Eur. Acad. Dermatol. Venereol* 32 (11), 1845–1851. doi: 10.1111/jdv.15146

Huang, J., Yin, C., and Wang, J. (2023). Relationship between vaginal microecological changes and oncogene E6/E7 and high-risk human papillomavirus infection. *J. Obstet Gynaecol* 43 (1), 2161349. doi: 10.1080/01443615.2022.2161349

Jahic, M., Mulavdic, M., Hadzimehmedovic, A., and Jahic, E. (2013). Association between aerobic vaginitis, bacterial vaginosis and squamous intraepithelial lesion of low grade. *Med. Arch.* 67 (2), 94–96. doi: 10.5455/medarh.2013.67.94-96

Kim, S. I., Yoon, J. H., Park, D. C., Lee, D. S., Lee, S. J., Choe, H. S., et al. (2018). Co-Infection of *Ureaplasma urealyticum* and human papilloma virus in asymptomatic sexually active individuals. *Int. J. Med. Sci.* 15 (9), 915–920. doi: 10.7150/ijms.26523

Klein, C., Samwel, K., Kahesa, C., Mwaiselage, J., West, J. T., Wood, C., et al. (2020). *Mycoplasma* Co-infection is associated with cervical cancer risk. *Cancers (Basel)* 12 (5):1093. doi: 10.3390/cancers12051093

Kombe Kombe, A. J., Li, B., Zahid, A., Mengist, H. M., Bounda, G. A., Zhou, Y., et al. (2020). Epidemiology and burden of human papillomavirus and related diseases, molecular pathogenesis, and vaccine evaluation. *Front. Public Health* 8. doi: 10.3389/fpubh.2020.552028

Li, S., and Wen, X. (2017). Seropositivity to herpes simplex virus type 2, but not type 1 is associated with cervical cancer: NHANES, (1999-2014). *BMC Cancer* 17 (1), 726. doi: 10.1186/s12885-017-3734-2

Ma, X., Wu, M., Wang, C., Li, H., Fan, A., Wang, Y., et al. (2022). The pathogenesis of prevalent aerobic bacteria in aerobic vaginitis and adverse pregnancy outcomes: a narrative review. *Reprod. Health* 19 (1), 21. doi: 10.1186/s12978-021-01292-8

Martins, B. C. T., Guimarães, R. A., Alves, R. R. F., and Saddi, V. A. (2023). Bacterial vaginosis and cervical human papillomavirus infection in young and adult women: a systematic review and meta-analysis. *Rev. Saude Publica* 56, 113. doi: 10.11606/s1518-8787.2022056004412

Naldini, G., Grisci, C., Chiavarini, M., and Fabiani, R. (2019). Association between human papillomavirus and *Chlamydia trachomatis* infection risk in women: a systematic review and meta-analysis. *Int. J. Public Health* 64 (6), 943–955. doi: 10.1007/s00038-019-01261-w

Ncib, K., Bahia, W., Leban, N., Mahdhi, A., Trifa, F., Mzoughi, R., et al. (2022). Microbial diversity and pathogenic properties of microbiota associated with aerobic vaginitis in women with recurrent pregnancy loss. *Diagnostics (Basel)* 12 (10):2444. doi: 10.3390/diagnostics12102444

Newman, L., Rowley, J., Vander Hoorn, S., Wijesooriya, N. S., Unemo, M., Low, N., et al. (2015). Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. *PloS One* 10 (12), e0143304. doi: 10.1371/journal.pone.0143304

Noma, I. H. Y., Shinobu-Mesquita, C. S., Suehiro, T. T., Morelli, F., De Souza, M. V. F., Damke, E., et al. (2021). Association of righ-risk human papillomavirus and *Ureaplasma parvum* Co-infections with increased risk of low-grade squamous intraepithelial cervical lesions. *Asian Pac J. Cancer Prev.* 22 (4), 1239–1246. doi: 10.31557/apjcp.2021.22.4.1239

Nugent, R. P., Krohn, M. A., and Hillier, S. L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J. Clin. Microbiol.* 29 (2), 297–301. doi: 10.1128/jcm.29.2.297-301.1991

World Health Organization (2021). *WHO guideline for screening and treatment of cervical pre-cancer lesions for cervical cancer prevention, 2nd ed.* Geneva: World Health Organization

Plisko, O., Zodzika, J., Jermakova, I., Pcolkina, K., Prusakevica, A., Liepniece-Karele, I., et al. (2021). Aerobic vaginitis-underestimated risk factor for cervical intraepithelial neoplasia. *Diagnostics (Basel)* 11 (1):97. doi: 10.3390/diagnostics11010097

Rittenschober-Böhm, J., Waldhoer, T., Schulz, S. M., Pimpel, B., Goeral, K., Kasper, D. C., et al. (2019). Vaginal *Ureaplasma parvum* serovars and spontaneous preterm birth. *Am. J. Obstet Gynecol* 220 (6), 594.e591–594.e599. doi: 10.1016/j.ajog.2019.01.237

Smith, J. S., Herrero, R., Bosetti, C., Muñoz, N., Bosch, F. X., Eluf-Neto, J., et al. (2002). Herpes simplex virus-2 as a human papillomavirus cofactor in the etiology of invasive cervical cancer. *J. Natl. Cancer Inst* 94 (21), 1604–1613. doi: 10.1093/jnci/94.21.1604

Solomon, D., Davey, D., Kurman, R., Moriarty, A., O'Connor, D., Prey, M., et al. (2002). The 2001 Bethesda system: terminology for reporting results of cervical cytology. *Jama* 287 (16), 2114–2119. doi: 10.1001/jama.287.16.2114

Tommasino, M. (2014). The human papillomavirus family and its role in carcinogenesis. *Semin. Cancer Biol.* 26, 13–21. doi: 10.1016/j.semcancer.2013.11.002

Tuddenham, S., Hamill, M. M., and Ghanem, K. G. (2022). Diagnosis and treatment of sexually transmitted infections: A review. *Jama* 327 (2), 161–172. doi: 10.1001/jama.2021.23487

Unemo, M., Bradshaw, C. S., Hocking, J. S., de Vries, H. J. C., Francis, S. C., Mabey, D., et al. (2017). Sexually transmitted infections: challenges ahead. *Lancet Infect. Dis.* 17 (8), e235–e279. doi: 10.1016/s1473-3099(17)30310-9

Usyk, M., Schlecht, N. F., Pickering, S., Williams, L., Sollecito, C. C., Gradissimo, A., et al. (2022). molBV reveals immune landscape of bacterial vaginosis and predicts human papillomavirus infection natural history. *Nat. Commun.* 13 (1), 233. doi: 10.1038/s41467-021-27628-3

Vieira-Baptista, P., Lima-Silva, J., Pinto, C., Saldanha, C., Beires, J., Martinez-de-Oliveira, J., et al. (2016). Bacterial vaginosis, aerobic vaginitis, vaginal inflammation and major pap smear abnormalities. *Eur. J. Clin. Microbiol. Infect. Dis.* 35 (4), 657–664. doi: 10.1007/s10096-016-2584-1

Wang, W., Zhang, X. H., Li, M., Hao, C. H., and Liang, H. P. (2020). Association between vaginal infections and the types and viral loads of human papillomavirus: A clinical study based on 4,449 cases of gynecologic outpatients. *Can. J. Infect. Dis. Med. Microbiol.* 2020, 9172908. doi: 10.1155/2020/9172908

Wang, L., Zhu, L., Li, H., Ma, N., Huang, H., Zhang, X., et al. (2019). Association between asymptomatic sexually transmitted infections and high-risk human papillomavirus in cervical lesions. *J. Int. Med. Res.* 47 (11), 5548–5559. doi: 10.1177/0300060519865633

Wright, T. C.Jr. (2017). The new ASCCP colposcopy standards. *J. Low Genit Tract Dis.* 21 (4), 215. doi: 10.1097/lgt.0000000000000337

Wu, M., Li, H., Yu, H., Yan, Y., Wang, C., Teng, F., et al. (2022). Disturbances of vaginal microbiome composition in human papillomavirus infection and cervical carcinogenesis: A qualitative systematic review. *Front. Oncol.* 12. doi: 10.3389/fonc.2022.941741

Xiao, L., Paralanov, V., Glass, J. I., Duffy, L. B., Robertson, J. A., Cassell, G. H., et al. (2011). Extensive horizontal gene transfer in *Ureaplasma*s from humans questions the utility of serotyping for diagnostic purposes. *J. Clin. Microbiol.* 49 (8), 2818–2826. doi: 10.1128/jcm.00637-11

Xie, L., Li, Q., Dong, X., Kong, Q., Duan, Y., Chen, X., et al. (2021). Investigation of the association between ten pathogens causing sexually transmitted diseases and high-risk human papilloma virus infection in shanghai. *Mol. Clin. Oncol.* 15 (1), 132. doi: 10.3892/mco.2021.2294

Xu, X., Rao, H., Fan, X., Pang, X., Wang, Y., Zhao, L., et al. (2023). HPV-related cervical diseases: Alteration of vaginal microbiotas and promising potential for diagnosis. *J. Med. Virol.* 95 (1), e28351. doi: 10.1002/jmv.28351

Zanotta, N., Campisciano, G., Morassut, S., Castro-Silva, E., Luksa, V., Zito, G., et al. (2019). Emerging role for *Ureaplasma parvum* serovar 3: Active infection in women with silent high-risk human papillomavirus and in women with idiopathic infertility. *J. Cell Physiol.* 234 (10), 17905–17911. doi: 10.1002/jcp.28423

Zeng, M., Li, X., Jiao, X., Cai, X., Yao, F., Xu, S., et al. (2022). Roles of vaginal flora in human papillomavirus infection, virus persistence and clearance. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.1036869

Zhang, D., and Liu, Z. (2016). Consensus on the diagnosis and treatment of mycopasma infection in genital tract. *Chin. J. Hum. Sexuality* 25 (03), 80–82.

Zhang, L. D., Pei, J., Zhang, H. M., and Sun, X. F. (2010). [Relationship between *Mycoplasma* and chlamydia infection and lesions in the cervical tissue in high-risk HPV-positive patients]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 24 (5), 346–348. doi: 10.3760/cma.j.issn.1003-9279.2010.05.009

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EDITED BY
Lei Cheng,
Biogas Institute of Ministry of Agriculture (CAAS),
China

REVIEWED BY
Bingbing Xiao,
Peking University,
China
Ruy Jauregui,
AgResearch Ltd,
New Zealand

*CORRESPONDENCE
Mengjun Luo
462816877@qq.com
Weixin Liu
liuweixind@163.com

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Association between the vaginal and uterine microbiota and the risk of early embryonic arrest

Li Wang[1], Junyu Chen[1], Lin He[1], Hanbo Liu[2], Yan Liu[2], Zonghui Luan[3], Hong Li[1], Weixin Liu[3]* and Mengjun Luo[1]*

[1]Department of Clinical Laboratory, School of Medicine, Chengdu Women's and Children's Central Hospital, University of Electronic Science and Technology of China, Chengdu, Sichuan, China, [2]Department of Gynecology, School of Medicine, Chengdu Women's and Children's Central Hospital, University of Electronic Science and Technology of China, Chengdu, Sichuan, China, [3]Key Laboratory of Reproductive Medicine, Sichuan Provincial Maternity and Child Health Care Hospital, The Affiliated Women's and Children's Hospital of Chengdu Medical College, Chengdu, Sichuan, China

The aim of this study was to explore the microecological distribution and differences in the uterus and vaginal microbiome in women with early embryonic arrest and those with normal pregnancy by high-throughput sequencing. We systematically sampled the vaginal and uterine microbiomes of 56 pregnant women, namely, 38 patients with early embryonic arrest and 18 pregnant women with normal pregnancy-induced abortion. We obtained colonization data by 16S rRNA gene amplicon sequencing. In the vagina, *Lactobacillus*, *Bacteroidetes* and *Helicobacter* exhibited significant differences between the groups. We further found that *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jensenii* were the most dominant *Lactobacillus* species and that *L. iners* was significantly different between the groups. Receiver operating characteristic (ROC) curve analysis confirmed that *Ensifer* had the highest predictive value for early embryonic arrest. In the uterine cavity, we determined that Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria were the dominant bacteria at the phylum level and that *Bacteroides*, *Pseudarthrobacter*, *Lactobacillus* and *Ralstonia* were the dominant genera. Further classification of *Lactobacillus* revealed that *L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii* were the main species. There was a significant difference in *L. jensenii* between the normal pregnancy group and early embryonic arrest group. Random forest analysis revealed 18 different genera in the uterus, and ROC curve analysis indicated that Candidatus Symbiobacter, Odoribacter, Blautia, Nocardioides and Ileibacterium had a certain predictive value.

KEYWORDS

embryo arrest, microbiome, pregnancy, vagina, uterus

Introduction

Early embryonic arrest is defined as the ultrasound diagnostic of an average diameter of the gestational sac diameter larger than 25 mm, no heartbeat, a crown-rump length exceeding 7 mm, and no fatal cardiac activity by the original ultrasound examination (Hendriks and MacKenzie, 2019). Early embryonic arrest is a component of early spontaneous abortion and a common complication in early pregnancy that can lead to adverse pregnancy outcomes. At present, the main treatment types for early embryonic developmental arrest are expectant management, medicinal management with mifepristone and misoprostol and uterine aspiration (Garcia-Grau

et al., 2019; Hendriks and MacKenzie, 2019). In recent years, the risk of embryonic arrest in women of childbearing age has increased, leading to widespread concern. Previous studies have reported that early embryonic arrest is related to chromosomal abnormalities, immunity, infection, endocrine dysfunction, environmental physical and chemical factors, among others. However, in 20–40% of cases, the causes are unknown (Fang et al., 2018). In recent years, studies have found that the occurrence of adverse pregnancy outcomes is significantly related to bacterial pathogens and other pathogenic microorganisms in the reproductive tract (Joseph Davey et al., 2016). In particular, the detection of Gardnerella vaginalis in vaginal secretions of patients with bacterial vaginosis (BV) is directly related to embryo arrest. The concentration of *G. vaginalis* in patients with embryo arrest is significantly higher than that in individuals without embryo arrest (Seo et al., 2017; Garcia-Grau et al., 2019).

The vaginal microbiota of women is complex, can change rapidly and dramatically and has a significant impact on women's health. As we all know, the vaginal secretions of women of normal childbearing age are dominated by *lactobacilli*, which account for more than 95% of the microbial content of the vagina (Antonio and Hillier, 1999). The protective bacteria in the vagina, can inhibit the excessive growth and reproduction of harmful anaerobic and aerobic bacteria, thus maintaining the microecological balance of the vagina. The application of probiotic *lactobacilli* in the clinical treatment and prevention of recurrent reproductive tract infections and vaginal microflora imbalance has become widespread (Chee et al., 2020). Therefore, the abundance and type of *lactobacilli* are important criteria for evaluating the imbalance of vaginal flora (Cohen et al., 2020). Vaginal flora imbalance caused by changes in the vaginal flora is closely related to diseases affecting not only the reproductive health of women during pregnancy but also the embryo. This may be because some infectious pathogens promote the growth of BV-related organisms from the lower genital tract, causing the destruction of the barrier and immune defense mechanism in the inflammatory environment (MacPhee et al., 2010).

With the development of next-generation sequencing (NGS), it has become evident that the endometrium not a sterile environment and that the species diversity and richness of the endometrial community are significantly higher than those of the vaginal environment (Verstraelen et al., 2016; Baker et al., 2018). The uterine microbiomes of women in reproductive age with the high abundance of *Lactobacillus* species, *Lactobacillus iners (L. iners)*, *Prevotella* spp. and *Lactobacillus crispatus (L. crispatus)*, and the endometrial bacteria are characterized by a polymicrobial ecosystem in the pregnant women (Mitchell et al., 2015; Riganelli et al., 2020). Changes to the abovementioned differences in the microbiome suggest that the intrauterine microbiome plays an important role in human health and diseases, and it helps to clarify the pathogenesis of some common gynecological and obstetrical diseases (Toson et al., 2022).

Previous literature has reported the isolation of vaginal microorganisms from the endometrium in 31.4% of women with acute salpingitis and 14.3% of women with pregnancy loss (den Heijer et al., 2019). Bacterial infection can activate the innate immune system, promote the synthesis of nitric oxide and prostaglandins and affect uterine receptivity and embryo implantation. Early embryo arrest not only affects the emotional and mental health of couples seeking to conceive but also causes serious reproductive tract infections. At present, the abundance of Lactobacillus is used to evaluate vaginal flora imbalance (Cohen et al., 2020). Prospective and retrospective cohort studies to assess the impact of vaginal and intrauterine microbiota on embryonic arrest are still lacking. Here, we aimed to identify microbiota differences among women with early embryo arrest and abortion, to improve the microbiota markers related to early embryo arrest.

Materials and methods

Study design

Samples were collected from December 2021 to April 2022 in the Obstetrics Clinic of Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China. 16S rRNA sequencing was performed on vaginal secretions and endometrial samples from 18 pregnant women with normal early pregnancy (N1–N18) and 38 patients with early pregnancy embryonic arrest (T1–T38). Both groups conceived naturally and needed negative pressure suction for artificial termination of pregnancy at 7–10 weeks of pregnancy. The inclusion criteria were as follows: (1) regular menstruation; (2) normal reproductive system development, no comorbidities such as immune diseases, chronic diseases or uterine organic lesions; and (3) no history of exposure to radioactivity or toxic substances, among others; (4) early pregnancy and the presence of a single intrauterine pregnancy sac by ultrasound; (5) exclusion of chromosomal disease in both parents and no family history of genetic conditions; (6) no obvious abnormalities by early laboratory testing, including positive indicators such as mycoplasma, chlamydia, syphilis, AIDS and hepatitis B virus; and (7) no history of sexual activity or vaginal lavage within 3 days before specimen collection, no history of antibiotic medication use in the previous 1 month, and no history of hormone medication in the previous 3 months.

Sample collection

All participants who met the experimental standards and signed an informed consent form received a gynecological examination conducted by a professional obstetrician. All samples were collected before artificial termination of pregnancy by negative pressure suction. Two sterile cotton swabs were used to collect secretions of the inner wall of the vagina. One of them was tested for the morphology and physicochemical properties of the vaginal secretions, and the other was stored at −80°C for 16S rRNA sequencing. Before the collection of endometrial specimens, a sterile speculum was inserted into the vagina so that the external orifice of the cervix could be seen. To minimize contamination of the vaginal wall and external cervical os with the endometrial microbiome, the cervix was cleaned with gauze soaked in iodine solution prior to endometrial biopsy. According to the literature (Verstraelen et al., 2016), we used an endometrial sampler, which uses a brush covered with plastic on the side and protected by plastic beads at the top to avoid contamination when passing through the vaginal cavity and cervix. After inserting the sheathed brush into the cervical tube, the brush was moved further upward to the uterine cavity, and the brush was pulled out. Then, the brush was rotated five times to collect a specimen from the surface of the entire endometrium. The brush was reshaped before it was removed from the uterine cavity. After the

TABLE 1 General data of the 56 included women.

Characteristics	Early embryonic arrest group (n =38)	Normal pregnancy group (n =18)	Value of p
Age (years)	30.68 ± 3.73	30.33 ± 4.74	*0.306*
Gestational age (weeks)	8.00 ± 1.77	7.28 ± 1.36	*0.511*
Number of pregnancies	3.26 ± 1.96	2.56 ± 1.62	*0.278*
Number of miscarriages	2.26 ± 1.93	1.33 ± 1.37	*0.208*

above procedure, the brush was aseptically separated from other parts of the device. Then, endometrial specimens were placed in sterile tubes and frozen at −80°C for 16S rRNA sequencing.

Vaginal discharge examination

Gram staining was performed on vaginal smears (Dai et al., 2010). After Gram staining, the following assessments were carried out: vaginal cleanliness, white blood cells, Lactobacillus counts, *Candida* spp. spores, blastospores and pseudohyphae, Trichomonas vaginalis counts, clue cell counts, bacterial density, flora diversity and dominant bacteria. Observations followed the National External Quality Assessment System (NSEQA) and College of American Pathologists (CAP) guidelines (Hu et al., 2015).

16S rRNA analysis

Sample gDNA purification was performed using the Zymo Research BIOMICS Microprep Kit. Specific primers were used to amplify the V4 region of the sample DNA. The NEW NEBNext UltraII DNA Library Prep Kit for Illumina (ENGLAND BioLabs) was used to build the library. The Illumina HiSeq Rapid SBS Kit v2 was used for high-throughput sequencing. FLASH was used to splice double-ended sequences. QIIME was used to separate each sample sequence from raw reads based on the barcode, and the barcode sequence was cut off. Based on Usearch software,[1] the UPARSE algorithm was used to cluster operational taxonomic units (OTUs) at a 97% consistency level, and the sequence with the highest frequency in each OTU was selected as the representative sequence of the OTU. To correct for differences in sequencing depth, we randomly subsampled the OTU table to a depth of 29,784 sequences per sample ten times before computing the alpha and beta diversity metrics. UCLUST taxonomy and the SILVA database were used for annotation analysis. Other diagrams were implemented using the R package.

Statistical analysis

The data were analyzed with SPSS statistical software SPSS 22.0 (IBM, Chicago, IL, United States). The results of normal distribution between groups are expressed as the mean ± standard deviation, and the samples were analyzed by t test. The Kruskal–Wallis test was used for the analysis of microbial differences. $p < 0.05$ indicated that the differences were statistically significant.

1 http://drive.com/uparse/

Results

The clinical characteristics of the pregnant women enrolled in this study are shown in Table 1. There was no significant difference in age between the early embryonic arrest and normal pregnancy groups. The gestational age, number of pregnancies and number of miscarriages of the fetuses were also not significantly different between the early embryonic arrest group and the normal pregnancy-induced abortion group ($p > 0.05$).

Comparison of vaginal microecology between the early embryo arrest and normal pregnancy groups

There was no significant difference in vaginal PH, vulvovaginal candidiasis (VVC) or trichomonas vaginitis (TV) between the early embryonic arrest group and the normal pregnancy-induced abortion group. However, the rates of bacterial vaginosis (BV) and vaginal microecological dysbiosis in the early embryonic arrest group were higher than those in the normal pregnancy induced-abortion group (Table 2).

Dysbiosis of the diversity of the vaginal microbiome in the early embryo arrest group

A total of 56 vaginal swab samples were analyzed by 16S rRNA gene sequencing to investigate the relationship between the embryo arrest group and normal pregnancy group. Among the samples, there were 38 vaginal swab samples in the early embryonic arrest group and 18 vaginal swab samples in the normal pregnancy group. A total of 1,921,346 reads were produced by an Illumina MiSeq, with an average of 34,309 reads per sample (shown in Supplementary Table 1). Based on the analysis of rarefaction curves of the two groups, it was found that the rarefaction curves of the

TABLE 2 Analysis of vaginal microecology in the early embryo arrest and normal pregnancy groups.

Microecological factors	Early embryonic arrest group case (%)	Normal pregnancy group case (%)	Value of p
Vaginal PH	4.49 ± 0.47	4.31 ± 0.53	*0.491*
BV	14 (36.84)	2 (11.11)	*0.047*
VVC	3 (7.89)	1 (5.55)	*0.751*
TV	0	0	–
Vaginal dysbiosis	24 (63.16)	6 (33.33)	*0.037*

TV, trichomonas vaginitis; VVC, vulvovaginal candidiasis, BV: bacterial vaginosis (BV).

two groups of vaginal swabs tended to be flat, indicating that the number of the sequences could cover all the data (shown in Supplementary Figure 1A). A total of 14,753 OTUs were obtained from 56 vaginal swab samples. Vaginal swabs from the early embryonic arrest group were associated with 10,027 OTUs, those from the control group were associated with 9,499 OTUs, and 4,726 OTUs were shared between the two groups (Figure 1A). The Chao1 index and PD whole tree measurements were calculated based on the OTU file to estimate within-sample α-diversity in the vaginal swabs. The Chao1 index (1227.69 ± 453.94 for the T group and 1834.60 ± 861.00 for the N group) and PD whole tree measurements (111.99 ± 26.43 for the T group and 150.04 ± 48.81 for the N group) of the vaginal swabs in the embryonic arrest group were significantly lower than those of the normal pregnancy group ($p < 0.05$; Figures 1B,C). Principal coordinate analysis based on the Bray–Curtis index ($p = 0.01$, $r = 0.13$) and the unweighted UniFrac index ($p = 0.001$, $r = 0.27$) exhibited significant differences in bacterial composition (Figures 1D,E).

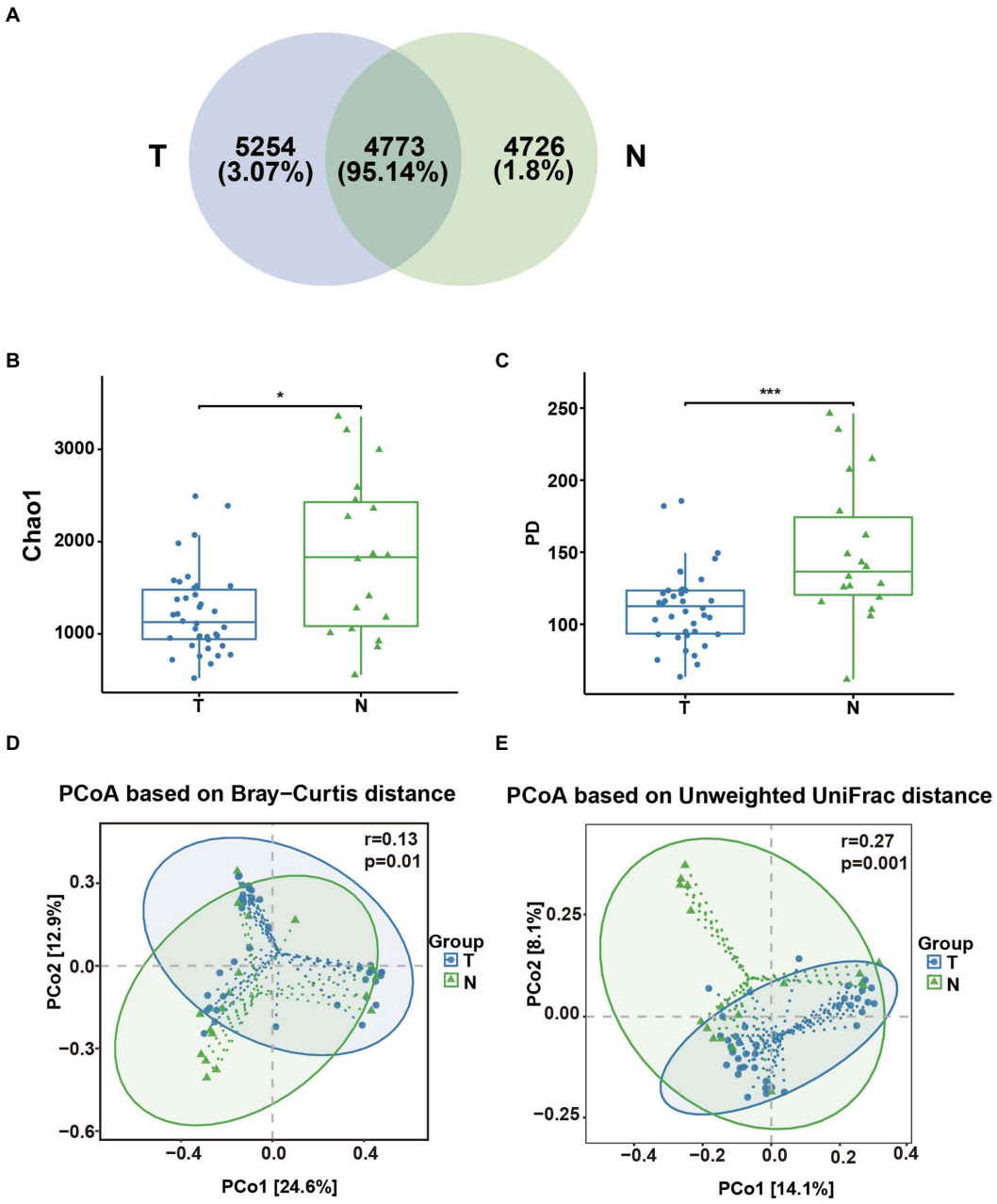

FIGURE 1
Analysis of the diversity of the vaginal microbial composition in the embryo-arrest and normal pregnancy groups. **(A)** Venn diagram of the numbers of operational taxonomic units (OTUs) identified in the two groups. **(B)** α-diversity of chao1. **(C)** α-diversity of the PD whole tree. **(D)** PCoA plot of the Bray–Curtis distance between the two groups. **(E)** PCoA plot of unweighted Uni-Frac distance between the two groups. T, embryo-arrest group; N, normal pregnancy group. Significance was tested with the Wilcoxon rank-sum test. *p<0.05; ***p<0.01.

Abundance changes in the vaginal microbiota between the early embryo arrest and normal pregnancy groups

The taxonomic classification at the phylum level showed similar patterns in the two groups, which were both dominated by Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. However, their relative abundances were not significantly different Figure 2A, ($p > 0.05$). At the genus level, the top 10 genera based on abundance are shown in Figure 2B. The abundance of *lactobacilli* in the normal pregnancy-induced abortion group was higher than that in the embryonic arrest group ($p < 0.05$). Accordingly, the abundance of *Bacteroides* and *Helicobacter* in the early embryonic arrest group was higher than that in the normal pregnancy-induced abortion group ($p < 0.05$). The abundances of *Lactobacillus*, *Gardnerella*, *Helicobacter*, *Prevotella 9*, *Ralstonia*, *Bacteroides* and *Pseudomonas* were higher than 1% in both the normal pregnancy and embryonic arrest groups (Figure 2B), while those of *Megasphaera*, *Pseudarthrobacter*, *Escherichia–Shigella*, *Sphingomonas*, and *Atopobium* were >1% only in the normal pregnancy-induced abortion group.

Discrete microbial communities showing significant differences

To determine whether specific vaginal bacteria were associated with an increased risk of early embryonic arrest, we used linear discriminant analysis effect size (LEfSe) analyses with a linear discriminant analysis (LDA) value of 4.0 at the genus level to identify specific microbial communities in both groups (Figure 3A). LEfSe analysis showed that compared with the normal pregnancy group, the abundance of *Bacteroides* and *Helicobacter* was significantly increased (Figure 3B). The *Lactobacillus* abundance in the normal pregnancy group was significantly higher than that in the embryonic arrest group (Figure 3A). We further analyzed the distribution of Lactobacillus at the species level. *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii* were the dominant bacteria (Figure 3B). There were significant differences in *L. iners* in the vaginal flora between the embryonic arrest group and the normal pregnancy group ($p < 0.05$). In the embryonic arrest group, the proportion of *L. iners* was significantly decreased (Figure 3B).

Operational taxonomic unit-based markers on vaginal swabs of embryo arrest

Based on operational taxonomic units (OTUs), the potential biomarkers of vaginal swabs for the predictive model of embryonic arrest occurrence were examined by the random forest algorithm among the ten different bacterial genera (Figure 4A). The target genera based on the Gini coefficient were predicted by receiver operating characteristic (ROC) curve analysis. In the patients diagnosed with embryo arrest, the area under the curve (AUC) of *Ensifer* and *Devosia* had the highest predictive value. The AUC of *Ensifer* was 0.82, and the AUC of *Devosia* was 0.80. The AUCs of *Bosea*, *Cellulomonas*, *Helicobacter* and *Sphingopyxis* had a certain predictive value (Figure 4B).

Dysbiosis and taxonomic alterations in the uterine microbiome profiles

Among the 56 intrauterine tissue samples, there were 38 intrauterine tissue samples in the early embryonic arrest group and 18 intrauterine tissue samples in the normal pregnancy group. A total of 1,920,598 reads were produced by Illumina MiSeq, with an average read count of approximately 34,296 (29,784-38,650; shown in Supplementary Table S1). Based on the analysis of the rarefaction

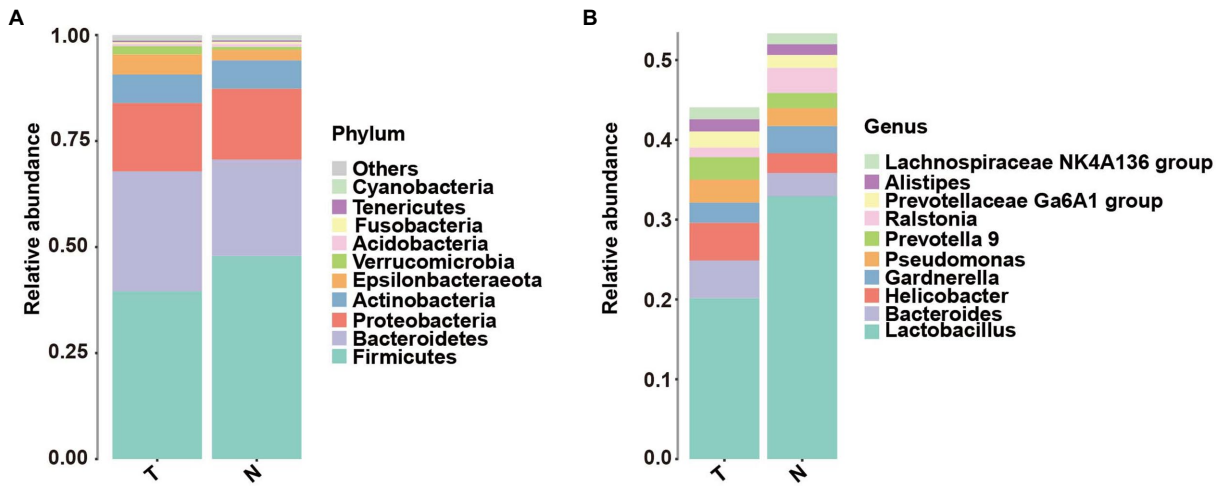

FIGURE 2
Abundance of microbes at different levels in the embryo-arrest and normal pregnancy groups. **(A)** Relative abundances of the vaginal microbiota at the phylum level. **(B)** Relative abundances of the vaginal microbiota at the genus level. T, embryo-arrest group; N, normal pregnancy group.

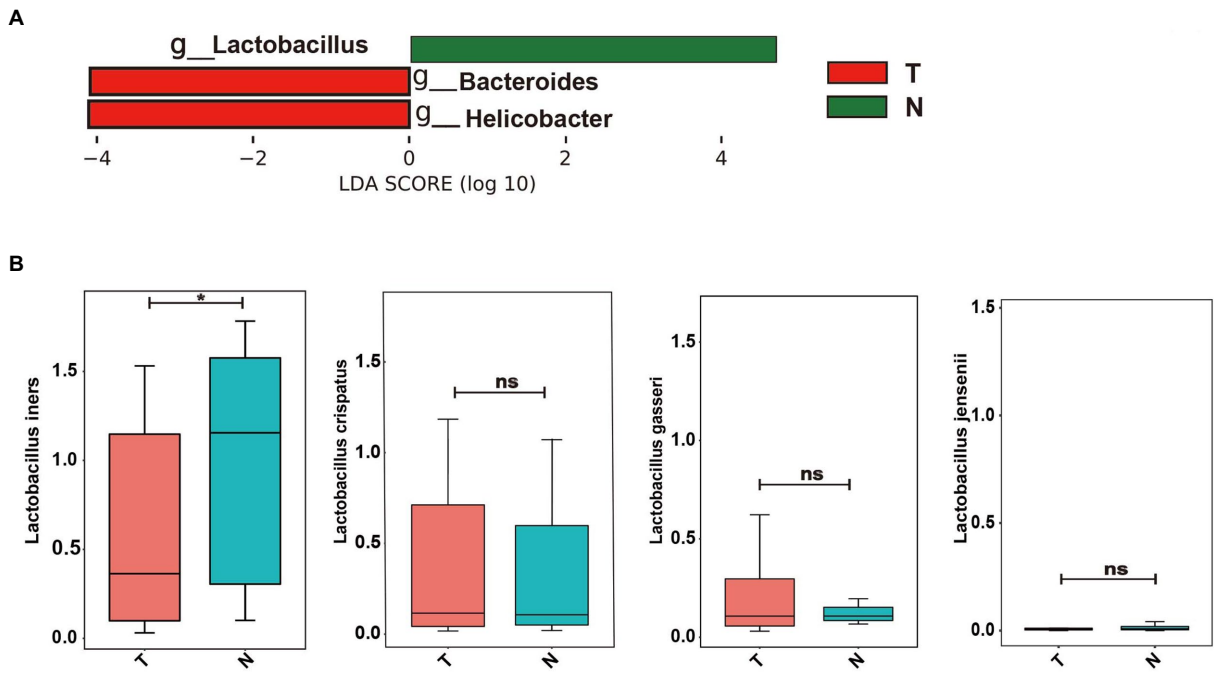

FIGURE 3
Bacterial taxa analysis of the embryo-arrest and normal pregnancy groups. **(A)** linear discriminant analysis (LDA) scores obtained from the linear discriminant analysis effect size (LEfSe) analysis of the vaginal microbiota in the two groups. **(B)** Relative abundance of *Lactobacillus* is reported with SEM as bar plots. An LDA effect size of >4 was used as a threshold for the LEfSe analysis. T, embryo-arrest group; N, normal pregnancy group. LDA, linear discriminant analysis; LEfSe, LDA effect size analysis.

curves of endometrial samples in the embryonic arrest group and the normal pregnancy group, it was found that the curve of the two groups tended to be flat, indicating that the number of sequences was sufficient (shown in Supplementary Figure S1B). There were a total of 8,169 OTUs in the uterine cavity group, 5,976 OTUs in the embryo-arrest group and 5,285 OTUs in the normal pregnancy group, which is similar to 3,082 OTUs, accounting for approximately 92.07% (Figure 5A). Based on the CHAO1 index and PD whole tree measurements, the α-diversity was evaluated, and there was no significant difference between the two groups ($p > 0.05$; Figures 5B,C). Based on the PCoA calculation of the Bray–Curtis distance and unweighted distance, there was no significant difference between the two groups of microbial communities ($p > 0.05$; Figures 5D,E).

Analysis of bacterial taxa in the uterine endometrium

Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and Epsilonbacteraeota were the dominant phyla in the endometrial microbiota in both groups (Figure 6A). At the genus level, *Bacteroides*, *Pseudarthrobacter*, *Lactobacillus*, *Ralstonia*, *Pseudomonas* and *Helicobacter* were the dominant microbial communities in both groups (Figure 6B). In the endometrium, *L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii* were the dominant groups of Lactobacillus among the uterine microbial bacteria (Figure 6C). The abundance of *L. jennsenii* was significantly different between the two groups ($p < 0.05$).

Operational taxonomic unit-based markers of embryo arrest in the vagina

The random forest analysis showed 18 genus differences between the two groups (Figure 7A). To evaluate the predictive power of endometrial microbiota for early embryonic arrest, we conducted ROC curve analysis of the six markers with significant differences between the two groups, and the data showed that the prediction value of the *Eubacterium xylanophilum group* was the highest. The AUC was 0.76. The AUCs of *Candidatus Symbiobacter*, *Odoribacter*, *Blautia*, *Nocardioides*, and *Ileibacterium* had a certain predictive value (Figure 7B).

Discussion

Embryonic arrest refers to the death of the embryo in early pregnancy, with an incidence of 10–15%, and 80% of miscarriages occur within the first 12 weeks of pregnancy (Peters et al., 2020). The incidence of early embryonic arrest has been increasing in recent years, and the high incidence of embryonic arrest has caused serious harm to many women and families (Fang et al., 2018). Therefore, determining the causes of embryonic arrest is the key to preventing the incidence of embryonic arrest from increasing. Previous studies have reported that in women with repeated embryo arrest, the relative abundance of *L. crispatus* in the endometrium was significantly lower than that in healthy women, while the abundance of *Gardnerella vaginalis* in the vagina and endometrium was significantly higher than that in healthy women. Changes in the

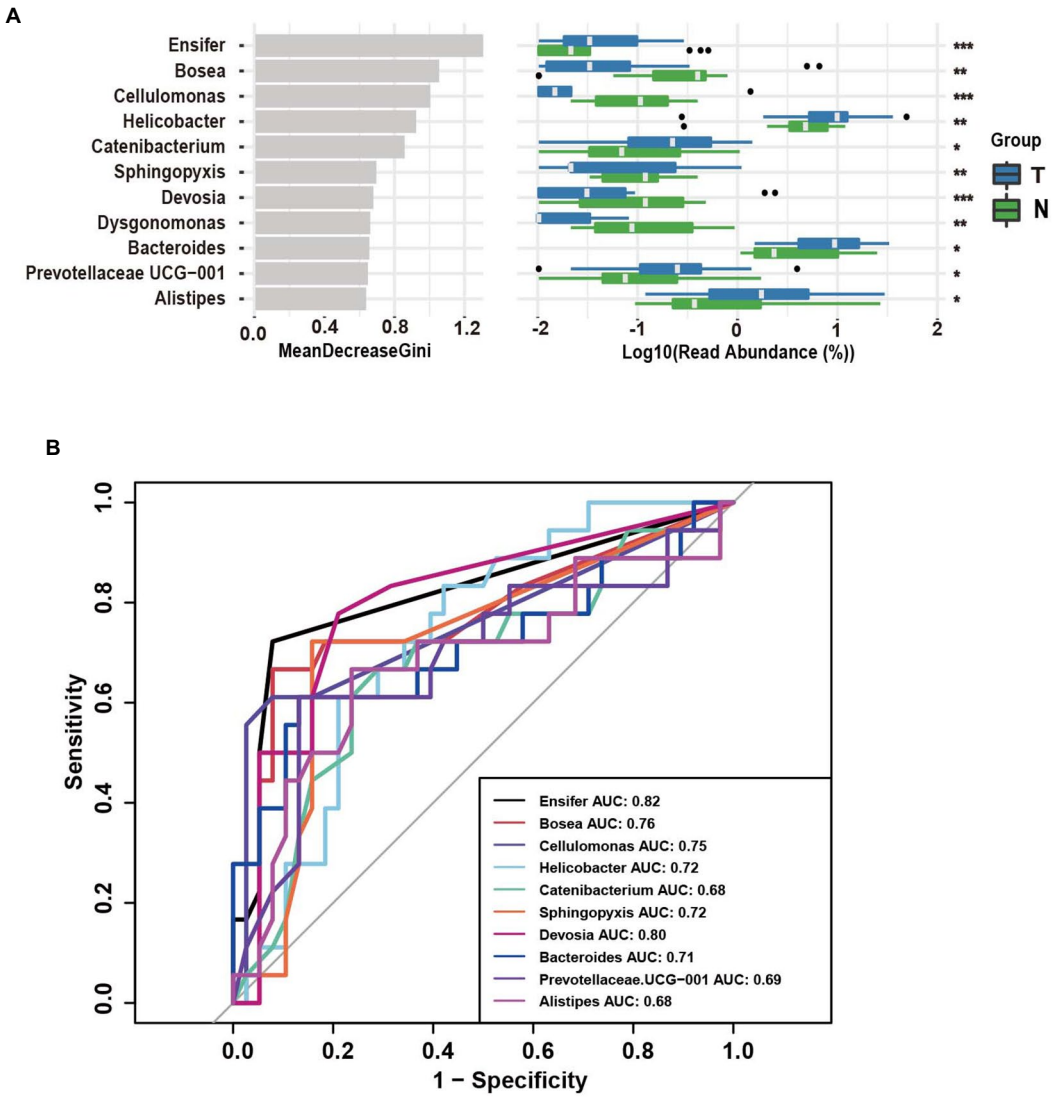

FIGURE 4
Operational taxonomic unit (OTUs)-based markers on vaginal swabs of the embryo-arrest and normal pregnancy groups. **(A)** Random forest algorithm of the vaginal microbiota in the two groups. **(B)** Receiver operating characteristic (ROC) curve for the prediction of embryo-arrest. T, embryo-arrest group; N, normal pregnancy group.

microecology of the reproductive tract are significantly associated with embryonic development and prognosis (Stout et al., 2017; Peuranpaa et al., 2022). Therefore, the development and prognosis of the reproductive tract microbiome and embryos have attracted increasing attention.

It is known that during pregnancy, due to the dramatic changes in estrogen and progesterone in women of childbearing age, the epithelial cells at the upper end of the vagina grow, and the production of lactic acid in the body increases, which results in changes in the reproductive tract, the pH and natural barriers of the vagina (Chen et al., 2021). The female genital tract accounts for approximately 9% of the human microbiome (Group NHW et al., 2009). Most previous research has focused on the vaginal microbiome, and less research has been conducted on the microbiome in the endometrium. However, dysbiosis of the endometrial microbiota was associated with lower rates of implantation, pregnancy, ongoing pregnancy, and increased spontaneous abortion obstetric complications (Garcia-Grau et al., 2019). The exact relationship between embryonic arrest and the reproductive tract microbiome is still unclear, and there are few correlation studies. Therefore, it is an urgent problem to determine the reason for embryonic arrest to reduce its risk.

Changes in reproductive tract bacteria during pregnancy are characterized by a decrease in the abundance of Lactobacillus and the diversity of the microflora (Di Simone et al., 2020). The most common clinical manifestation of dysbacteriosis during pregnancy is BV of the reproductive tract (Kovachev, 2018; Jayaram et al., 2020). BV is closely related to perinatal maternal and infant health, such as premature rupture of membranes and preterm delivery (Kaambo, 2017; Juliana et al., 2020). In our study, the incidence of BV in the embryo arrest group was significantly higher than that in the normal pregnancy group. In recent years, the clinical incidence of embryo arrest has increased, and some related studies have confirmed that it is closely related to the microflora of the reproductive tract. In the pathogenesis of BV, which is characterized

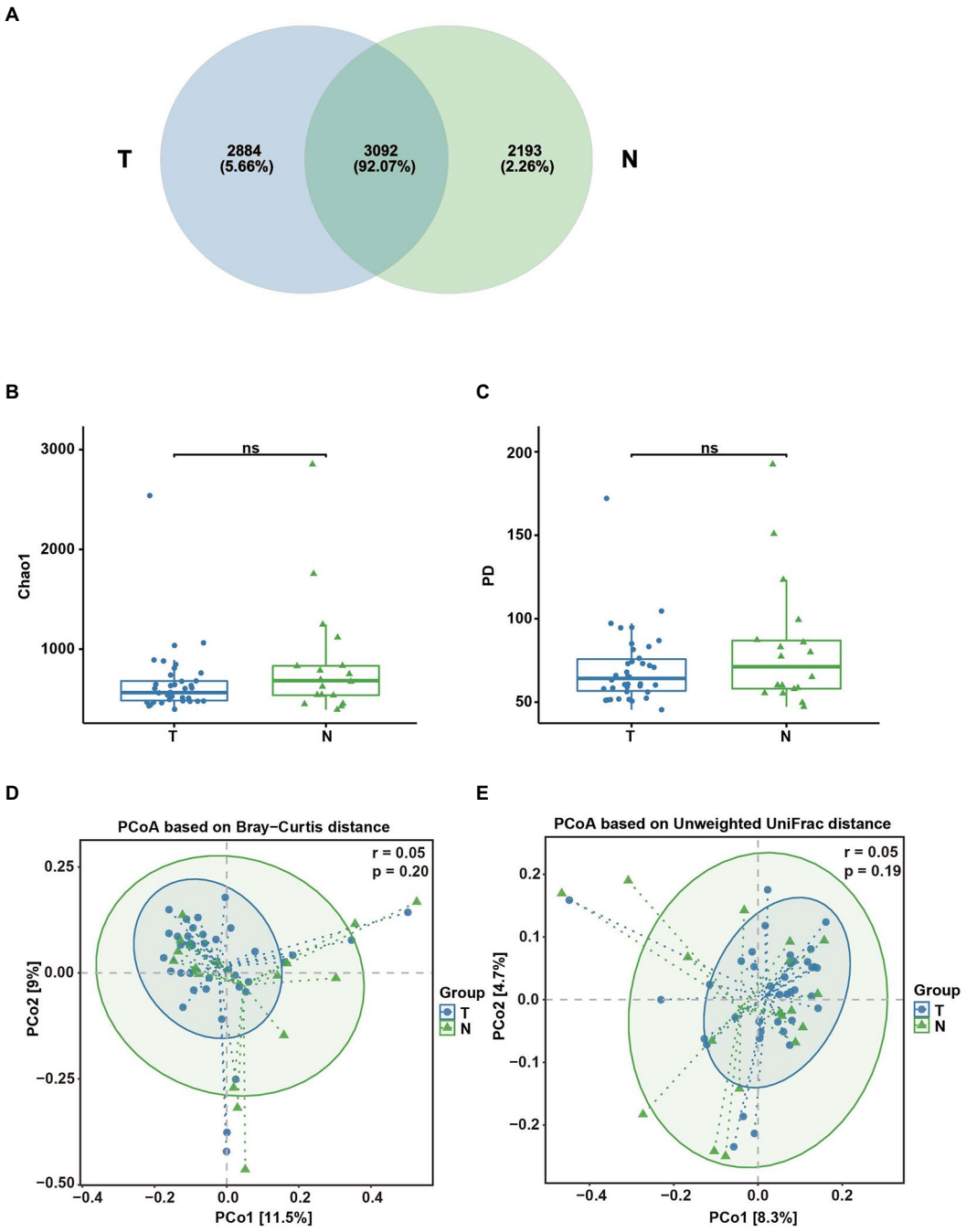

FIGURE 5
Analysis of the diversity of the uterine microbial composition in the embryo-arrest and normal pregnancy groups. **(A)** Venn diagram of the numbers of OTUs identified in the two groups. **(B)** α-diversity of chao1. **(C)** α-diversity of the PD whole tree. **(D)** PCoA plot of the Bray–Curtis distance between the two groups. **(E)** PCoA plot of unweighted Uni-Frac distance between the two groups. T, embryo-arrest group; N, normal pregnancy group. Significance was tested with the Wilcoxon rank-sum test.

by a decrease in Lactobacillus vaginalis and an increase in Gardnerella, there is no characteristic inflammatory change in the vagina (Yan et al., 2016). Therefore, we further analyzed vaginal swabs for Lactobacillus and Gardnerella. To our surprise, the abundance of Lactobacillus decreased significantly in the embryo-arrest group (shown in Figure 2). However, there was no significant difference in the abundance of Gardnerella. Based on the above research, we speculate that the abundance of Lactobacillus may be more closely related to embryo arrest, which is also consistent with previous studies (Al-Memar et al., 2019).

The dominant genus Lactobacillus in the vagina of women of childbearing age is a protective bacterium of the vaginal microbiome (Kalia et al., 2020). In patients undergoing *in vitro* fertilization (IVF), the success rate of IVF is higher if only Lactobacillus is present in the vagina at different time points. Lactobacillus may play a certain role in pregnancy, embryo colonization and normal

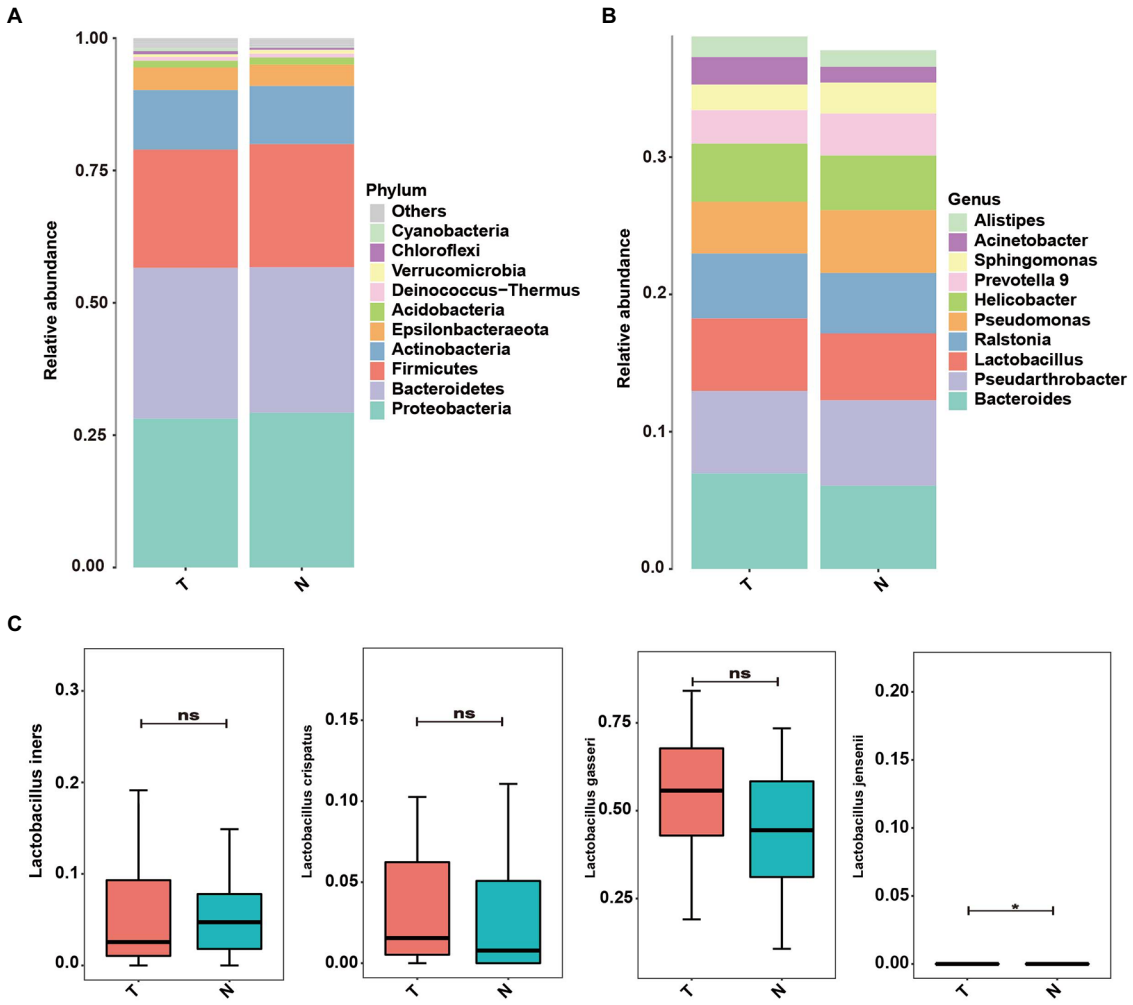

FIGURE 6
Abundance of microbes at different levels in the uterus of embryo-arrest and normal pregnancy groups. **(A)** Relative abundances of the vaginal microbiota at the phylum level. **(B)** Relative abundances of the vaginal microbiota at the genus level. **(C)** Relative abundance of *Lactobacillus* was reported with SEM as bar plots. T, embryo-arrest group; N, normal pregnancy group.

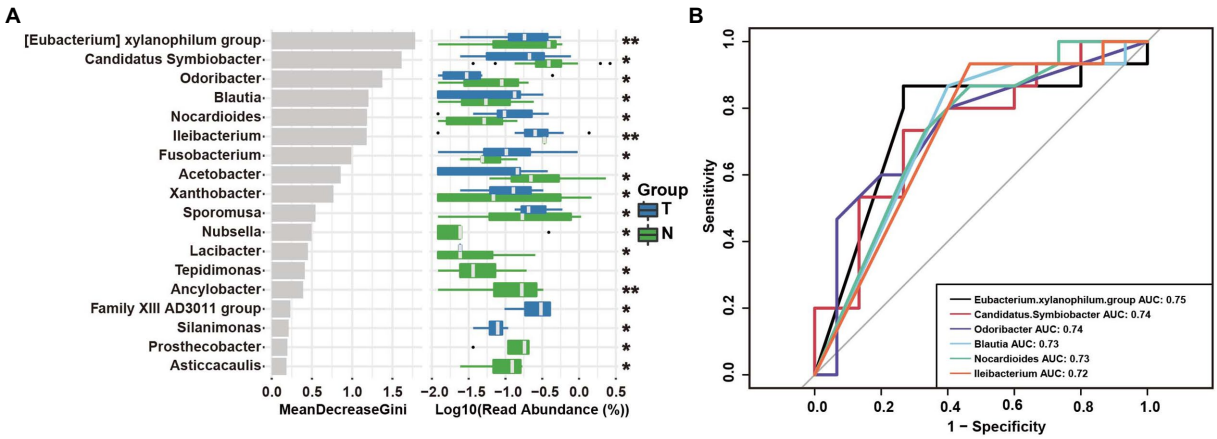

FIGURE 7
Operational taxonomic unit (OTU)-based markers in the uterus of the embryo-arrest and normal pregnancy groups. **(A)** Random forest algorithm of the uterine microbiota in the two groups. **(B)** Receiver operating characteristics (ROC) curve for the prediction of embryo-arrest. T, embryo-arrest group; N, normal pregnancy group.

development during pregnancy (Leitich and Kiss, 2007; Hyman et al., 2012). Previous studies have also confirmed that the bacteria in the female vagina are dominated mainly by lactobacillus species, namely, *L. crispatus*, *L. iners*, *L. jensenii*, and *L. gasseri* (Romero et al., 2014; DiGiulio et al., 2015; Witkin et al., 2019, 2021). The vaginal microbiome of healthy women has been classified into five community state types (CSTs) according to their community structure. Four types of CST are dominated by Lactobacillus, namely *L. crispatus* (CST I), *L. gasseri* (CST II), *L. Iners* (CST III) and *L. jenesii* (CST V). CST IV is characterized by low levels or absence of *Lactobacillus* (Ravel et al., 2011; Liu et al., 2021). Previous studies have reported that *lactobacilli* in the vagina during pregnancy are dominated mainly by *L. crispatus* (CST I). In addition, the higher the abundance of *L. crispatus* is, the lower the abundance of other *Lactobacillus* species will be (Witkin et al., 2021). To explore the role of the community state types (CSTs) of Lactobacillus in embryonic development, we further classified *Lactobacillus* in the vagina. In our study, both groups were dominated by *L. iners* (CST III), but the abundance of *L. iners* in the embryo-arrest group was significantly lower than that in the normal pregnancy group (shown in Figure 3). At present, research on *L. iners* is still controversial, and its exact function in the vagina is uncertain. Studies have confirmed that *L. iners* is a beneficial species and is related to vaginal dynamic balance. It can survive and help restore vaginal homeostasis (Ferris et al., 2004; Petrova et al., 2017). In the transitional stage of the vaginal microbiota, it may become the dominant species related to term delivery in pregnant women and associated with a high risk of adverse pregnancy outcomes (Jakobsson and Forsum, 2007; Romero et al., 2014; Stout et al., 2017). Our research shows that the balance of vaginal microbiology may play a certain role in the occurrence of embryo arrest and that domination of the internal environment by different lactobacilli would have different effects vaginal health in women.

At present, the study of the vaginal microbiome is still the focus of research, and an increasing number of studies have focused on changes in the vaginal microbiome during pregnancy, to infer its impact on pregnancy outcomes. Previous studies have confirmed that the abundance of Lactobacillus increases and that the diversity of bacteria decreases during pregnancy. In our study, the diversity of bacteria in the embryo arrest group decreased significantly, and Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria were dominant in the two groups at the phylum level; however, at the genus level, the abundance of Lactobacillus in the embryo arrest group decreased significantly, and the corresponding Bacteroides and Helicobacter increased significantly. To identify the bacteria related to embryo arrest in the vagina of the embryo arrest group and the normal pregnancy group, we used random forest analysis to identify the different bacteria and further predict embryo arrest by ROC curve analysis. We found that *Ensifer* and *Bosea* had high values in embryo arrest. In this study, we detected two special strains, Ensifer and Bosea. Ensifer was isolated in 1982 and located in the soil. Previously, scholars have reported that Ensifer microbes may be temporary in the skin, and have been found to be present in human skin for hours to days (Suwarsa et al., 2021). Bosea was detected in cervical microflora in a study of the relationship between human papillomavirus infection and age, and it was significantly associated with HPV infection (Hu et al., 2022). Interestingly, these two strains were detected in the vagina of pregnant women with embryo arrest and normal pregnant women, their abundances were low, and there was a significant difference between the two groups; however, their specific effects need to be further studied. The development of next-generation sequencing techniques has improved our understanding of the vaginal microbiota, which represents a unique microbiota (Franasiak et al., 2016; Verstraelen et al., 2016).

For a long time, research on the microbiota of the female reproductive tract during pregnancy has focused mainly on the vagina. Due to the limited collection of intrauterine samples during pregnancy, there are few studies on intrauterine flora during pregnancy (Moreno and Simon, 2018; Riganelli et al., 2020). Previous studies have shown that bacteria can enter and reproduce in the uterus from the vagina and cervix (Ramos Bde et al., 2015). The changes in the endometrial microbiome, characterized by a low abundance of lactobacilli, seem to be related to the maintenance and implantation of embryos. However, the current reports of the microbiota in the uterus vary widely. Due to the low abundance of the microbiome in the uterus, fewer changes in the vagina and uterus, and the possibility of sample contamination is inevitable (Baker et al., 2018). The new study is still exploring the interaction between the microbial profiles in the uterine cavity and the host during pregnancy. At present, the study of the uterine microbiome still needs to overcome some technical challenges to avoid misleading conclusions. The abortion caused by the stagnation of early embryonic development usually occurs in the early stage of pregnancy, so the changes in microflora in the uterine cavity of pregnant women have an important impact on the changes in embryos. We further attempted to use high-throughput sequencing to discover the unique microbiota in the uterine cavity of patients with embryonic arrest and women during normal pregnancy. The bacterial diversity of in the uterine cavity was not significantly different between the embryo-arrest group and normal pregnancy group. The core assemblage microbiota at the phylum level were Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria similar to the vaginal microbiota. However, the abundances were significantly different from that of the vaginal microbiome. The microbiota in the uterus has the highest proportion of Proteobacteria, and the highest proportion of Firmicutes in the vagina (Toson et al., 2022). At the genus level, we observed that *Bacteroides*, *Pseudarthrobacter*, *Lactobacillus*, *Ralstonia* and *Pseudomonas* accounted for the main proportion between the embryo-arrest group and the normal pregnancy group. This is consistent with previous studies reporting that non-*Lactobacillus* was the dominant genus (Mei et al., 2019; Moreno et al., 2020). However, it has also confirmed that the microbiome in the uterus is not dominated by lactobacilli (Leoni et al., 2019). The bacteria in the endometrium mentioned above not only include Lactobacillus, but are also significantly related to pregnancy.

There is a certain relationship between the imbalance in endometrial microbiota and the determination of the cause of infertility, and changes in the microbiome of may be the reason for disease in women (van Oostrum et al., 2013; Moreno and Simon, 2018). The cervix forms a complete barrier between the uterus and vagina. The mucins in the cervix change their conformation, allowing bacteria to pass through under certain conditions (Brunelli et al., 2007). It has been confirmed that there is a significant correlation between the abundance of lactobacilli and pregnancy outcomes in

recurrent pregnancy loss. The abundance of Lactobacillus was significantly correlated with implantation, pregnancy and live birth rate in women. The survival rate of embryos is higher when the microbiome of the uterine cavity comprises Lactobacillus-dominant microbiota at the time of implantation, and pregnancy (Moreno et al., 2016; Shi et al., 2022). However, infection of the pathogenic microbiome in the reproductive tract, especially inflammatory reactions, destroys the structure of the chorion and amniotic membrane, which is toxic to the endometrium and the normal development of embryos, making this an important cause of embryo arrest (Giakoumelou et al., 2016). In our study, we explored the relationship between the endometrial microbiome and pregnancy outcomes and observed significant changes in the intrauterine cavity microbiome in the embryo-arrest group and the normal pregnancy group. We further classified the Lactobacillus, and found that the Lactobacillus in the uterus is also dominated by *L. iners*, *L. crispatus*, *L. gasseri* and *L. jensenii*. To our surprise, *L. jensenii in utero* was significantly different between the embryo-arrest group and normal pregnancy groups. In the vagina, the abundance of the above microbiota is significantly related to preterm delivery. In pregnant women with missed abortion, this abundance is significantly decreased (Sun et al., 2022). The exact role of Lactobacillus species in the uterine cavity has not been reported thus far and its exact effect on embryo arrest remains to be explored. More research is still needed on the relationship between the microflora in the uterine cavity and embryo arrest in order to characterize the different microbiomes *in utero* and evaluate their predictive value for embryo arrest. Through random forest analysis, we analyzed the ROC curves of the bacteria with significant differences in the uterus. We found that the *Eubacterium xylanophilum group*, Candidatus Symbiobacter, Odoribacter, Blautia, *Nocardioies* and *Ileibacterium* have a certain predictive value for embryo arrest. Thus, the specific mechanism of the above bacteria in the implantation and outcome of embryos in the uterus as well as the interactions of bacteria in the uterine cavity need to be further studied.

Thus, it can be seen that the balance of vaginal microecology and uterine microflora during pregnancy is very important for the development of embryos. There are many reports on the balance of microecology in the vagina during pregnancy, but it is also affected by a variety of factors, including the interaction of bacteria to maintain the relative balance of the vagina. Embryo arrest is a complex process, not only in the vaginal environment, but also in the uterine cavity. Our study explored the effect of the environment in the reproductive tract on embryo arrest from two aspects of the difference in vaginal and uterine flora, to provide some research ideas for the prevention of embryo termination.

Conclusion

With the support of high-throughput sequencing technology, we performed a detailed study on the uterine and vaginal microbiomes of patients with embryo arrest and women with normal pregnancies. There were significant differences in the uterine and vaginal microbiomes between these two groups. The above study of the microbiome provides insights into the pathogenesis of embryo arrest and, according to our evidence. Additionally, our findings indicate that some bacteria have a certain predictive value for embryo arrest. However, due to the limitation of sample size and specimen collection, the predictive value of the microbiome in the uterus and vagina for embryo termination is limited, and further prospective studies are needed to determine the predictive value of the microbiome.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA933192.

Ethics statement

The studies involving human participants were reviewed and approved by Chengdu Women's and Children's Central Hospital (grant no. 2021(88)). The patients/participants provided their written informed consent to participate in this study.

Author contributions

LW and ML contributed to the study design. LH, JC, HLiu, and YL completed all tests. LW, ML and WL analyzed the data and drafted this paper. HLi and ZL contributed to reagent management. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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References

Al-Memar, M., Bobdiwala, S., Fourie, H., Mannino, R., Lee, Y. S., Smith, A., et al. (2019). The association between vaginal bacterial composition and miscarriage: a nested case–control study. *BJOG Int. J. Obstet. Gynaecol.* 127, 264–274. doi: 10.1111/1471-0528.15972

Antonio, M. A. H. S., and Hillier, S. L. (1999). The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J. Infect. Dis.* 180, 1950–1956. doi: 10.1086/315109

Baker, J. M., Chase, D. M., and Herbst-Kralovetz, M. M. (2018). Uterine microbiota: residents, tourists, or invaders? *Front. Immunol.* 9:208. doi: 10.3389/fimmu.2018.00208

Brunelli, R., Papi, M., Arcovito, G., Bompiani, A., Castagnola, M., Parasassi, T., et al. (2007). Globular structure of human ovulatory cervical mucus. *FASEB J.* 21, 3872–3876. doi: 10.1096/fj.07-8189com

Chee, W. J. Y., Chew, S. Y., and Than, L. T. L. (2020). Vaginal microbiota and the potential of lactobacillus derivatives in maintaining vaginal health. *Microb. Cell Factories* 19:203. doi: 10.1186/s12934-020-01464-4

Chen, X., Lu, Y., Chen, T., and Li, R. (2021). The female vaginal microbiome in health and bacterial vaginosis. *Front. Cell. Infect. Microbiol.* 11:631972. doi: 10.3389/fcimb.2021.631972

Cohen, C. R., Wierzbicki, M. R., French, A. L., Morris, S., Newmann, S., Reno, H., et al. (2020). Randomized trial of Lactin-V to prevent recurrence of bacterial vaginosis. *N. Engl. J. Med.* 382, 1906–1915. doi: 10.1056/NEJMoa1915254

Dai, Q., Hu, L., Jiang, Y., Shi, H., Liu, J., Zhou, W., et al. (2010). An epidemiological survey of bacterial vaginosis, vulvovaginal candidiasis and trichomoniasis in the Tibetan area of Sichuan Province, China. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 150, 207–209. doi: 10.1016/j.ejogrb.2010.02.027

den Heijer, C. D. J., Hoebe, C., Driessen, J. H. M., Wolffs, P., van den Broek, I. V. F., Hoenderboom, B. M., et al. (2019). Chlamydia trachomatis and the risk of pelvic inflammatory disease, ectopic pregnancy, and female infertility: a retrospective cohort study among primary care patients. *Clin. Infect. Dis.* 69, 1517–1525. doi: 10.1093/cid/ciz429

Di Simone, N., Santamaria Ortiz, A., Specchia, M., Tersigni, C., Villa, P., Gasbarrini, A., et al. (2020). Recent insights on the maternal microbiota: impact on pregnancy outcomes. *Front. Immunol.* 11:528202. doi: 10.3389/fimmu.2020.528202

DiGiulio, D. B., Callahan, B. J., McMurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., et al. (2015). Temporal and spatial variation of the human microbiota during pregnancy. *Proc. Natl. Acad. Sci. U. S. A.* 112, 11060–11065. doi: 10.1073/pnas.1502875112

Fang, J., Xie, B., Chen, B., Qiao, C., Zheng, B., Luan, X., et al. (2018). Biochemical clinical factors associated with missed abortion independent of maternal age: a retrospective study of 795 cases with missed abortion and 694 cases with normal pregnancy. *Medicine (Baltimore)* 97:e13573. doi: 10.1097/MD.0000000000013573

Ferris, M. J. M. A., Aldridge, K. E., Fortenberry, J. D., Fidel, P. L. Jr., and Martin, D. H. (2004). Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. *BMC Infect. Dis.* 4:5.doi: 10.1186/1471-2334-4-5.

Franasiak, J. M., Werner, M. D., Juneau, C. R., Tao, X., Landis, J., Zhan, Y., et al. (2016). Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. *J. Assist. Reprod. Genet.* 33, 129–136. doi: 10.1007/s10815-015-0614-z

Garcia-Grau, I., Perez-Villaroya, D., Bau, D., Gonzalez-Monfort, M., Vilella, F., Moreno, I., et al. (2019). Taxonomical and functional assessment of the endometrial microbiota in a context of recurrent reproductive failure: a case report. *Pathogens* 8:8. doi: 10.3390/pathogens8040205

Giakoumelou, S., Wheelhouse, N., Cuschieri, K., Entrican, G., Howie, S. E., and Horne, A. W. (2016). The role of infection in miscarriage. *Hum. Reprod. Update* 22, 116–133. doi: 10.1093/humupd/dmv041

Group NHWPeterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., et al. (2009). The NIH human microbiome project. *Genome Res.* 19, 2317–2323. doi: 10.1101/gr.096651.109

Hendriks, E. M. H., and MacKenzie, M. C. (2019). First trimester bleeding: evaluation and management. *Am. Fam. Physician* 99, 166–174.

Hu, Z. Z. W., Mu, L., Kuang, L., Su, M., and Jiang, Y. (2015). Identification of cytolytic vaginosis versus vulvovaginal candidiasis. *J. Low. Genit. Tract Dis.* 19, 152–155. doi: 10.1097/LGT.0000000000000076

Hu, J., Wu, Y., Quan, L., Yang, W., Lang, J., Tian, G., et al. (2022). Research of cervical microbiota alterations with human papillomavirus infection status and women age in Sanmenxia area of China. *Front. Microbiol.* 13:1004664. doi: 10.3389/fmicb.2022.1004664

Hyman, R. W., Herndon, C. N., Jiang, H., Palm, C., Fukushima, M., Bernstein, D., et al. (2012). The dynamics of the vaginal microbiome during infertility therapy with in vitro fertilization-embryo transfer. *J. Assist. Reprod. Genet.* 29, 105–115. doi: 10.1007/s10815-011-9694-6

Jakobsson, T., and Forsum, U. (2007). *Lactobacillus iners*: a marker of changes in the vaginal flora? *J. Clin. Microbiol.* 45:3145. doi: 10.1128/JCM.00558-07

Jayaram, P. M., Mohan, M. K., and Konje, J. (2020). Bacterial vaginosis in pregnancy: a storm in the cup of tea. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 253, 220–224. doi: 10.1016/j.ejogrb.2020.08.009

Joseph Davey, D. L., Shull, H. I., Billings, J. D., Wang, D., Adachi, K., and Klausner, J. D. (2016). Prevalence of curable sexually transmitted infections in pregnant women in low- and middle-income countries from 2010 to 2015: a systematic review. *Sex. Transm. Dis.* 43, 450–458. doi: 10.1097/OLQ.0000000000000460

Juliana, N. C. A., Suiters, M. J. M., Al-Nasiry, S., Morre, S. A., Peters, R. P. H., and Ambrosino, E. (2020). The association between vaginal microbiota Dysbiosis, bacterial vaginosis, and aerobic vaginitis, and adverse pregnancy outcomes of women living in sub-Saharan Africa: a systematic review. *Front. Public Health* 8:567885. doi: 10.3389/fpubh.2020.567885

Kaambo, E. A. C. (2017). The threat of aerobic vaginitis to pregnancy and neonatal morbidity. *Afr. J. Reprod. Health* 21, 108–118. doi: 10.29063/ajrh2017/v21i2.12

Kalia, N., Singh, J., and Kaur, M. (2020). Microbiota in vaginal health and pathogenesis of recurrent vulvovaginal infections: a critical review. *Ann. Clin. Microbiol. Antimicrob.* 19:5. doi: 10.1186/s12941-020-0347-4

Kovachev, S. (2018). Defence factors of vaginal lactobacilli. *Crit. Rev. Microbiol.* 44, 31–39. doi: 10.1080/1040841X.2017.1306688

Leitich, H., and Kiss, H. (2007). Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. *Best Pract. Res. Clin. Obstet. Gynaecol.* 21, 375–390. doi: 10.1016/j.bpobgyn.2006.12.005

Leoni, C., Ceci, O., Manzari, C., Fosso, B., Volpicella, M., Ferrari, A., et al. (2019). Human endometrial microbiota at term of Normal pregnancies. *Genes (Basel)* 10:10. doi: 10.3390/genes10120971

Liu, X., Cao, Y., Xie, X., Qin, X., He, X., Shi, C., et al. (2021). Association between vaginal microbiota and risk of early pregnancy miscarriage. *Comp. Immunol. Microbiol. Infect. Dis.* 77:101669. doi: 10.1016/j.cimid.2021.101669

MacPhee, R. A. H. R., Bisanz, J. E., Miller, W. L., and Reid, G. (2010). Probiotic strategies for the treatment and prevention of bacterial vaginosis. *Expert. Opin. Pharmacother.* 11, 2985–2995. doi: 10.1517/14656566.2010.512004

Mei, C., Yang, W., Wei, X., Wu, K., and Huang, D. (2019). The unique microbiome and innate immunity during pregnancy. *Front. Immunol.* 10:2886. doi: 10.3389/fimmu.2019.02886

Mitchell, C. M., Haick, A., Nkwopara, E., Garcia, R., Rendi, M., Agnew, K., et al. (2015). Colonization of the upper genital tract by vaginal bacterial species in nonpregnant women. *Am. J. Obstet. Gynecol.* 212, 611.e1–611.e9. doi: 10.1016/j.ajog.2014.11.043

Moreno, I., Codoner, F. M., Vilella, F., Valbuena, D., Martinez-Blanch, J. F., Jimenez-Almazan, J., et al. (2016). Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am. J. Obstet. Gynecol.* 215, 684–703. doi: 10.1016/j.ajog.2016.09.075

Moreno, I., Garcia-Grau, I., Bau, D., Perez-Villaroya, D., Gonzalez-Monfort, M., Vilella, F., et al. (2020). The first glimpse of the endometrial microbiota in early pregnancy. *Am. J. Obstet. Gynecol.* 222, 296–305. doi: 10.1016/j.ajog.2020.01.031

Moreno, I., and Simon, C. (2018). Relevance of assessing the uterine microbiota in infertility. *Fertil. Steril.* 110, 337–343. doi: 10.1016/j.fertnstert.2018.04.041

Peters, S. A. E., Yang, L., Guo, Y., Chen, Y., Bian, Z., Sun, H., et al. (2020). Pregnancy, pregnancy loss and the risk of diabetes in Chinese women: findings from the China Kadoorie biobank. *Eur. J. Epidemiol.* 35, 295–303. doi: 10.1007/s10654-019-00582-7

Petrova, M. I., Reid, G., Vaneechoutte, M., and Lebeer, S. (2017). *Lactobacillus iners*: friend or foe? *Trends Microbiol.* 25, 182–191. doi: 10.1016/j.tim.2016.11.007

Peuranpaa, P., Holster, T., Saqib, S., Kalliala, I., Tiitinen, A., Salonen, A., et al. (2022). Female reproductive tract microbiota and recurrent pregnancy loss: a nested case-control study. *Reprod. Biomed. Online* 45, 1021–1031. doi: 10.1016/j.rbmo.2022.06.008

Ramos Bde, A., Kanninen, T. T., Sisti, G., and Witkin, S. S. (2015). Microorganisms in the female genital tract during pregnancy: tolerance versus pathogenesis. *Am. J. Reprod. Immunol.* 73, 383–389. doi: 10.1111/aji.12326

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U. S. A.* 108, 4680–4687. doi: 10.1073/pnas.1002611107

Riganelli, L., Iebba, V., Piccioni, M., Illuminati, I., Bonfiglio, G., Neroni, B., et al. (2020). Structural variations of vaginal and endometrial microbiota: hints on female infertility. *Front. Cell. Infect. Microbiol.* 10:350. doi: 10.3389/fcimb.2020.00350

Romero, R. H. S., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., et al. (2014). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2:4. doi: 10.1186/2049-2618-2-4

Romero, R. H. S., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Bieda, J., et al. (2014). The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. *Microbiome* 2:18. doi: 10.1186/2049-2618-2-18

Seo, S. S., Arokiyaraj, S., Kim, M. K., Oh, H. Y., Kwon, M., Kong, J. S., et al. (2017). High prevalence of *Leptotrichia amnionii*, *Atopobium vaginae*, *Sneathia sanguinegens*,

and factor 1 microbes and Association of spontaneous abortion among Korean women. *Biomed. Res. Int.* 2017:5435089. doi: 10.1155/2017/5435089

Shi, Y., Yamada, H., Sasagawa, Y., Tanimura, K., and Deguchi, M. (2022). Uterine endometrium microbiota and pregnancy outcome in women with recurrent pregnancy loss. *J. Reprod. Immunol.* 152:103653. doi: 10.1016/j.jri.2022.103653

Stout, M. J., Zhou, Y., Wylie, K. M., Tarr, P. I., Macones, G. A., and Tuuli, M. G. (2017). Early pregnancy vaginal microbiome trends and preterm birth. *Am. J. Obstet. Gynecol.* 217, 356.e1–356.e18. doi: 10.1016/j.ajog.2017.05.030

Sun, D., Zhao, X., Pan, Q., Li, F., Gao, B., Zhang, A., et al. (2022). The association between vaginal microbiota disorders and early missed abortion: a prospective study. *Acta Obstet. Gynecol. Scand.* 101, 960–971. doi: 10.1111/aogs.14410

Suwarsa, O., Hazari, M. N., Dharmadji, H. P., Dwiyana, R. F., Effendi, R. M. R. A., Hidayah, R. M. N., et al. (2021). A pilot study: composition and diversity of 16S rRNA based skin bacterial microbiome in Indonesian atopic dermatitis population. *Clin. Cosmet. Investig. Dermatol.*, 1737–1744. doi: 10.2147/ccid.S338550

Toson, B., Simon, C., and Moreno, I. (2022). The endometrial microbiome and its impact on human conception. *Int. J. Mol. Sci.* 23:23. doi: 10.3390/ijms23010485

van Oostrum, N., De Sutter, P., Meys, J., and Verstraelen, H. (2013). Risks associated with bacterial vaginosis in infertility patients: a systematic review and meta-analysis. *Hum. Reprod.* 28, 1809–1815. doi: 10.1093/humrep/det096

Verstraelen, H., Vilchez-Vargas, R., Desimpel, F., Jauregui, R., Vankeirsbilck, N., Weyers, S., et al. (2016). Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. *PeerJ* 4:e1602. doi: 10.7717/peerj.1602

Witkin, S. S., Moron, A. F., Linhares, I. M., and Forney, L. J. (2021). Influence of *Lactobacillus crispatus*, *Lactobacillus iners* and *Gardnerella vaginalis* on bacterial vaginal composition in pregnant women. *Arch. Gynecol. Obstet.* 304, 395–400. doi: 10.1007/s00404-021-05978-z

Witkin, S. S., Moron, A. F., Ridenhour, B. J., Minis, E., Hatanaka, A., Sarmento, S. G.P., et al. (2019). Vaginal biomarkers that predict cervical length and dominant bacteria in the vaginal microbiomes of pregnant women. *MBio* 10, e02242–e02219. doi: 10.1128/mBio

Yan, S. F., Liu, X. Y., Cheng, Y. F., Li, Z. Y., Ou, J., Wang, W., et al. (2016). Relationship between intrauterine bacterial infection and early embryonic developmental arrest. *Chin. Med. J.* 129, 1455–1458. doi: 10.4103/0366-6999.183411

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EDITED BY
Zhangran Chen,
Shenzhen Wedge Microbiology Research Co. Ltd,
China

REVIEWED BY
Simone Filardo,
Sapienza University of Rome,
Italy
Zhiguang Zhou,
Second Xiangya Hospital,
Central South University,
China
Tesfaye Belay,
Bluefield State College,
United States
Yimou Wu,
University of South China,
China

*CORRESPONDENCE
Qi Tian
✉ qi-tian@hotmail.com
Tianyuan Zhang
✉ zty1911@126.com

†These authors have contributed equally to this work and share first authorship

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Gut dysbiosis contributes to chlamydial induction of hydrosalpinx in the upper genital tract

Qi Tian[1,2*†], Tianyuan Zhang[3*†], Luying Wang[4], Jingyue Ma[5] and Xin Sun[4]

[1]Department of Obstetrics and Gynecology, Hunan Provincial Maternal and Child Health Care Hospital, Changsha, Hunan, China, [2]National Health Commission Key Laboratory for Birth Defect Research and Prevention, Hunan Provincial Maternal and Child Health Care Hospital, Changsha, Hunan, China, [3]Key Lab of Molecular Virology and Immunology, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China, [4]Department of Obstetrics and Gynecology, Third Xiangya Hospital, Central South University, Changsha, Hunan, China, [5]Department of Dermatovenereology, Tianjin Medical University General Hospital, Tianjin, China

Chlamydia trachomatis is one of the most common sexually infections that cause infertility, and its genital infection induces tubal adhesion and hydrosalpinx. Intravaginal *Chlamydia muridarum* infection in mice can induce hydrosalpinx in the upper genital tract and it has been used for studying *C. trachomatis* pathogenicity. DBA2/J strain mice were known to be resistant to the chlamydial induction of hydrosalpinx. In this study, we took advantage of this feature of DBA2/J mice to evaluate the role of antibiotic induced dysbiosis in chlamydial pathogenicity. Antibiotics (vancomycin and gentamicin) were orally administrated to induce dysbiosis in the gut of DBA2/J mice. The mice with or without antibiotic treatment were evaluated for gut and genital dysbiosis and then intravaginally challenged by *C. muridarum*. Chlamydial burden was tested and genital pathologies were evaluated. We found that oral antibiotics significantly enhanced chlamydial induction of genital hydrosalpinx. And the antibiotic treatment induced severe dysbiosis in the GI tract, including significantly reduced fecal DNA and increased ratios of firmicutes over bacteroidetes. The oral antibiotic did not alter chlamydial infection or microbiota in the mouse genital tracts. Our study showed that the oral antibiotics-enhanced hydrosalpinx correlated with dysbiosis in gut, providing the evidence for associating gut microbiome with chlamydial genital pathogenicity.

KEYWORDS

Oral antibiotics, gut dysbiosis, *Chlamydia muridarum*, genital tract, pathology

Introduction

Chlamydia trachomatis can cause long-lasting genital fibrosis and may result in tubal infertility (Strandell et al., 1994; Vasquez et al., 1995; Land et al., 2010; Budrys et al., 2012; Jansen et al., 2016), but the mechanism of the process remains poorly understood. Mouse genital infection with *Chlamydia muridarum* was a widely used model to investigate chlamydial pathogenesis. Tubal inflammation caused by chlamydial ascension was defined as a key pathogenic factor in this model (Rodgers et al., 2010, 2011; Budrys et al., 2012; Chen et al., 2014; Lei et al., 2014; Liu et al., 2014; Yang et al., 2014; Zhong, 2017). Many chlamydial (Liu et al., 2014; Huang et al., 2015) and host (Natividad et al., 2007; Asquith et al., 2011; Li et al., 2012; Andrew et al., 2013; Dong et al., 2014; Yang et al., 2014; Manam et al., 2015) factors were identified to affect the chlamydial ascension (Chen et al., 2010; Frazer et al., 2013) and tubal inflammation (Cheng et al., 2008; Murthy et al.,

2011; Yang et al., 2014). Interestingly, not all mice with *C. muridarum* ascension and tubal inflammation may develop hydrosalpinx (Chen et al., 2014). In human cases, only a portion of women with high titers of serum anti-Chlamydia antibodies develop long-term complications (Rodgers et al., 2010, 2011; Budrys et al., 2012). These observations suggested possible extra-genital mechanisms in the development of genital pathology during chlamydia infection.

Studies showed that genital microbiota played a role in the host susceptibility to chlamydial infection (Nelson et al., 2010; Ravel et al., 2011; Bai et al., 2012; Hickey et al., 2012; Ma et al., 2012; Ravel and Brotman, 2016; Noyes et al., 2018; van Houdt et al., 2018). *Lactobacillus iners* dominated genital or cervicovaginal microbiota was associated with high susceptibility to chlamydial infection while *L. crispatus* promoted resistance (Nardini et al., 2016; van Houdt et al., 2018). It is assumed that certain genital microbiota may cooperate with *C. trachomatis* (Ziklo et al., 2016) and help it to evade the inhibition of IFN-γ by taking up indole from Prevotella (Ravel et al., 2011; Ziklo et al., 2016). Although the genital microbiome is associated with chlamydial ascending infection, the causative correlation between these two remains unclear. And the role of gut microbiome in chlamydial pathogenicity has not been investigated. It has been shown that gut microbiota may regulate fibrosis process in the development of extra-gut pathologies by inducing profibrotic regulatory T cells (Metwali et al., 2006; Owyang et al., 2006; Koch and Muller, 2015; Tai et al., 2016; Shivaji, 2017). We hypothesized that gut microbiota might be able to impact chlamydial pathogenicity in the upper genital tract considering the fact that fibrosis is a key feature of chlamydial pathogenicity (Murthy et al., 2007; Chen et al., 2009; Rodgers et al., 2010, 2011; Peng et al., 2011; Budrys et al., 2012; Li et al., 2012; Lu et al., 2012; Chen et al., 2014; Lei et al., 2014; Liu et al., 2014; Tang et al., 2014; Yang et al., 2014; Yadav et al., 2017; Zhang et al., 2014; Zhong, 2017).

The DBA2/J mice were known to resist the development of hydrosalpinx following an intravaginal infection with *C. muridarum* (Chen et al., 2014). In the current study, oral antibiotic (vancomycin and gentamicin, ABX) cocktails were given to DBA2/J mice to cause gut dysbiosis and the mice were further intravaginally challenged by *C. muridarum* to induce hydrosalpinx. Since the vancomycin and gentamicin cannot bypass eukaryotic cell membrane or affect chlamydial infection (Ridgway et al., 1978; Dailloux et al., 1990), it allowed us to evaluate the effect of the ABX-induced gut dysbiosis on chlamydial pathogenicity selectively. We found that the oral ABX indeed promoted chlamydial induction of hydrosalpinx significantly without changing the chlamydial infection courses in the lower or upper genital tract. The ABX caused severe dysbiosis in the gastrointestinal (GI) tract, including significantly reduced fecal DNA and increased ratios of firmicutes over bacteroidetes but ABX did not change vaginal microbiota greatly. Furthermore, the oral antibiotics did not affect *C. muridarum* colonization in the GI tract either.

The current study has provided the experimental evidence for associating gut microbiome with chlamydial pathogenicity. This study may also provide a platform to study the impacts of gut microbiome on genital tract pathogenesis.

Materials and methods

The growth of chlamydial organism

The *Chlamydia muridarum* derived from Mouse Pneumonitis strain Nigg II (ATCC #VR-123) was used in this study. The *Chlamydia muridarum* organisms were grown in HeLa cells and purified as elementary bodies as previously described (Fan et al., 1998; Zhang et al., 2015). The purified EBs were stored in aliquots at -80° C until use.

Mice and treatment

DBA2/J mice (6–7 weeks old female) were purchased from Vital River, Beijing. Mice were bred and maintained under specific pathogen free (SPF) conditions in the institutional animal facility of the Institute of Pasteur of Shanghai Chinese Academy of Sciences. Mice were treated with or without a cocktail of vancomycin (A100990-0001, Sangon Biotech, Shanghai) and gentamicin (A620217-0025, Sangon Biotech, Shanghai) *via* oral gavage on days 1 and 2. The ABX cocktail was then maintained in drink water in the ABX group until mice were sacrificed. For oral gavage, vancomycin and gentamicin were used 3 mg/ml each in a total volume of 200ul per dosing per mouse. 0.5 mg/ml each of vancomycin and gentamicin were used in drinking water. Twenty-one days after the ABX treatment, mice were intravaginally challenged with 2×10^5 IFUs of *Chlamydia muridarum*. Vaginal and rectal swabs were taken on days 3 and 7 post and then on a weekly basis thereafter. Specifically, on each sampling occasion, one vaginal swab and one rectal swab were obtained from each mouse for analysis. The swabs were further analyzed for live chlamydial organisms. Tissue chlamydial organisms were measured in some experiments by tissue homogenization.

Titrating live chlamydial organisms recovered from swabs and tissue homogenates

To quantitate live chlamydial organisms in vaginal or rectal swabs, each swab was immersed in 0.5 ml of iced SPG and vortexed with glass beads in 1.5 ml tube (Chen et al., 2015). The tube was centrifuged and the chlamydial organisms in the supernatants were then titrated on HeLa cell monolayers. The infected cell cultures were further processed for immunofluorescence assay as previously described (Kapoor et al., 2023). For each titration, the inclusions were counted under a fluorescence microscope and five random fields were counted for each coverslip. The total number of IFUs per sample was calculated according to the mean IFUs per view, the ratio of the view area to the well, dilution ratio, and volumes. The total number of IFUs/swab was then converted by log10 and the final number presented the chlamydial burden of each mouse at each time point. To quantitate chlamydial organisms in tissues, the tissue (or organ) was homogenized in 0.5 to 5 ml cold SPG depending on the sizes with an automatic homogenizer [Omni Tissue Homogenizer, TH115, Kennesaw, GA]. The homogenates were sonicated and spun at 3000 rpm for 5 min. The supernatants were titrated and counted for live *C. muridarum* organisms the same as the swabs and as previously described (Lei et al., 2014).

Immunofluorescence assay

The primary antibody: A rabbit antibody (made with purified *C. muridarum* elementary bodies) was gift from Jingyue Ma in Tianjin Medical University General Hospital. After primary antibody incubation, the secondary antibody (goat anti-rabbit IgG conjugated with Cy2,

#ab6940, Abcam) was added and the nuclei was visualized by DNA dye Hoechst 33258 (#ab228550, Abcam). The doubly labeled samples were used for counting for chlamydial inclusions under a fluorescence microscope (AX70, Olympus) with a CCD camera (Hamamatsu).

Evaluation of genital macroscopical pathology

Seventy days after *C. muridarum* infection, mice were sacrificed for evaluating genital pathology. The upper genital tract hydrosalpinx was evaluated. Before the genital tract was removed, an *in situ* gross examination was made for evidence of oviduct hydrosalpinx.

The oviduct hydrosalpinx score was made based on the following criteria: no hydrosalpinx (0 score), hydrosalpinx detectable only under microscope (1 score), hydrosalpinx visible with naked eyes but the size was smaller than the ovary on the same side (2 score), equal to the ovary on the same side (3 score) or larger than the ovary on the same side (4 score). Scores from both sides of the oviduct from the same mouse were added up to represent the score for a given mouse. The individual mouse scores were calculated into means ± standard errors.

Fecal microbiota evaluation

Fecal samples were collected from female DBA2/J mice both before and after the oral ABX treatment. The fecal samples were frozen in liquid nitrogen and stored at -80°C until DNA extraction. The fecal genomic DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen Inc., Germantown, MD20874) according to the manufacturers' instructions. The purity and quantity of DNA samples were determined by absorbance ratios at 260/280. A real time/quantitative PCR (iTaq universal SYBRgreen super mix 500, Bio-Rad) was used to quantitate bacterial 16S rRNA genes in each sample. The following PCR condition was used: 95°C x 5 min for denaturation, then 95°C x 5 s, 60°C x 30s to 90s for 45 cycles as described previously (Yang et al., 2015). The primers specific for bacterial phyla Bacteroidetes and Firmicutes and a pair of universal primers are shown in Table 1. These primers were adopted from the published studies elsewhere (Bacchetti De Gregoris et al., 2011; Yang et al., 2015).

Statistics analyses

The number of live organisms expressed as IFUs, Log10 IFUs, 16S rRNA gene copies. The chlamydial genome copies and hydrosalpinx scores were compared between groups using Wilcoxon rank sum test.

TABLE 1 The primers specific for bacterial phyla Bacteroidetes and Firmicutes and a pair of universal primers.

Firmicutes F	GGAGYATGTGGTTTAATTCGAAGCA
Firmicutes R	AGCTGACGACAACCATGCAC
Bacteroidetes F	GTTTAATTCGATGATACGCGAG
Bacteroidetes R	TTAASCCGACACCTCACGG
Universal F	AAACTCAAAKGAATTGACGG
Universal R	CTCACRRCACGAGCTGAC

The incidences of hydrosalpinx between groups were evaluated by Fisher's Exact Probability Test.[1]

Correlations of chlamydial pathogenicity in the upper genital tract with chlamydial colonization in different tissues were analyzed by calculating Spearman Rank-Order Correlation Coefficients.[2] Furthermore, the Significance of the Difference Between Two Correlation Coefficients was calculated.[3]

Results

Oral ABX treatment significantly increases the susceptibility of hydrosalpinx induction by *Chlamydia muridarum* intravaginal infection in DBA2/J mice

The previous study has showed that DBA2/J mice are naturally resistant to hydrosalpinx induction by intravaginal infection with *C. muridarum* (Chen et al., 2014). Here we used an oral administration of membrane-impermeable antibiotics vancomycin and gentamicin cocktail to induce gut dysbiosis in DBA2/J mice. As shown in Figure 1, female DBA2/J mice without the ABX treatment

1 http://vassarstats.net/tab2x2.html
2 http://vassarstats.net/corr_rank.html
3 http://vassarstats.net/rdiff.html

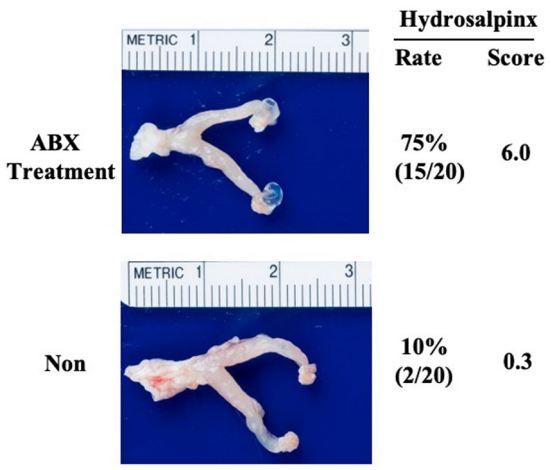

FIGURE 1
Oral ABX treatment significantly increases the susceptibility of hydrosalpinx induction by *C. muridarum* intravaginal infection in DBA2/J mice. Female DBA2/J mice without (Non, *n*=20) or with (ABX, *n*=20) treatment with vancomycin and gentamicin cocktail (ABX) for 21days were intravaginally inoculated with a live *C. muridarum* clone (G13.32.1). The oral ABX was maintained in drinking water throughout the experiment. On day 70 after the intravaginal inoculation, all mice were sacrificed for evaluating genital tract pathology. Hydrosalpinx from each mouse was evaluated macroscopically under naked eye as described in the Materials and Methods. One representative genital tract image from each group was shown with the vagina on the left and oviduct/ovary on the right corresponding hydrosalpinx scores marked. Data came from 3 independent experiments. Note that ABX treatment significantly increased pathology rate and severity macroscopically. Fisher's Exact for comparing rates and Wilcoxon rank sum for scores.

remained highly resistant to hydrosalpinx induction by *C. muridarum*, and the hydrosalpinx incidence rate was only 10% and a mean severity score was ~0.3. Only two out of 20 mice developed minimal levels of hydrosalpinx in 3 independent experiments. The lack of oviduct pathology was confirmed by microscopic evaluation of oviduct dilation and inflammatory infiltration. However, in the group of ABX treatment, the hydrosalpinx incidence increased to >70% with a mean severity score of ~6. A total of 15 out of 20 mice treated with the oral ABX cocktail developed significant hydrosalpinx. The evidence of significant oviduct dilation and tubal inflammatory infiltration was microscopically confirmed in these mice. As a result, we have demonstrated that oral administration with vancomycin and gentamicin is sufficient to promote chlamydial pathogenicity in the upper genital tract of DBA2/J mice.

Oral ABX treatment did not alter chlamydial infection or microbiota in the genital tract

It has been shown that the altered microbiota and enhanced chlamydial infection may impact the chlamydial pathogenicity (Chen et al., 2014; Ziklo et al., 2016). We evaluated the chlamydial infection or microbiota in the mice with or without ABX treatment. As shown in Figure 2, significant live organism shedding was similarly detected in all mice up to day 28 after the intravaginal infection regardless of the oral ABX treatment. The level of shedding and number of mice remaining positive for live organisms were both similar between mice with or without ABX treatment. Since the live organism shedding could only reflect chlamydial infections in the lower genital tract, we further tested the chlamydial infection by tissue homogenization of upper genital tract (Figure 3). On day 14 after intravaginal inoculation, the day of infection reaches the highest level, the mice were sacrificed to evaluate the live organism distribution across the whole genital tract. We recovered high levels of live chlamydial organisms from both sides of uterine horns and oviducts regardless of the oral ABX treatment. The oral ABX cocktail did not affect genital chlamydial ascension.

To further evaluate the effect of the oral antibiotics on genital microbiota, we compared the microbiota in vaginal swabs between mice with or without oral ABX treatment (Figure 4). We found that the amounts of DNA were similar from vaginal swabs regardless of the oral ABX treatment. More importantly, when 16S rRNA gene copy was quantitated using qPCR with primers targeting bacterial phyla bacteroidetes and firmicutes, the relative abundance of these two phyla was similar between mice with or without oral ABX treatment for 21 days (prior to infection) or 91 days (70 days after chlamydial infection). Thus, the oral ABX used in the current study did not significantly alter the microbiota in the genital tract.

Oral antibiotics treatment induces dysbiosis without affecting *Chlamydia muridarum* colonization in the GI tract

Since genital *C. muridarum* has been shown to spread to and colonize in the GI tract (Zhang et al., 2015) and the GI*C. muridarum* has been proposed to promote chlamydial pathogenicity in the upper genital tract (Zhong, 2018), we next evaluated the effect of the oral ABX on the chlamydial colonization (Figure 5) and distribution (Figure 6) in the GI tract. We found that the oral ABX cocktail did not affect the overall colonization of *C. muridarum* in the GI tracts up to 70 days after intravaginal infection. Mice with or without oral ABX treatment maintained relatively steady and similar infection time courses. On day 14 after intravaginal *C. muridarum* infection, some mice were sacrificed for evaluating the distribution of *C. muridarum*, we found that similar levels of live *C. muridarum* organisms were recovered from different gut tissues of all these mice regardless of the oral ABX treatment. Thus, chlamydial colonization and distribution in the GItract were not significantly altered by the oral ABX treatment. We then compared the fecal microbiota between mice with or without oral ABX treatment (Figure 7).

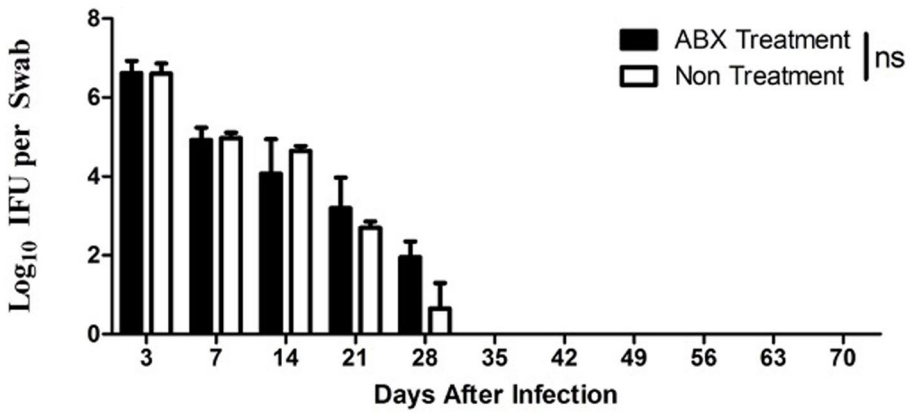

FIGURE 2
Oral ABX treatment did not alter chlamydial infection in the genital tract. Female DBA2/J mice without (Non, *n*=20) or with (ABX, *n*=20), oral antibiotics treatment for 21days was intravaginally infected with *C. muridarum* as described in Figure 1 legend. Live organism shedding was monitored from vaginal swabs on days 3, 7 and weekly thereafter (up to day 70) as shown along X-axis. Both live organisms recovered (Log10 IFUs per swab) were shown along *Y*-axis. Note that significant shedding was similarly detected in all mice up to day 28 after infection regardless of the oral antibiotics' treatment ($p>0.05$, Wilcoxon for comparing area-under-curve).

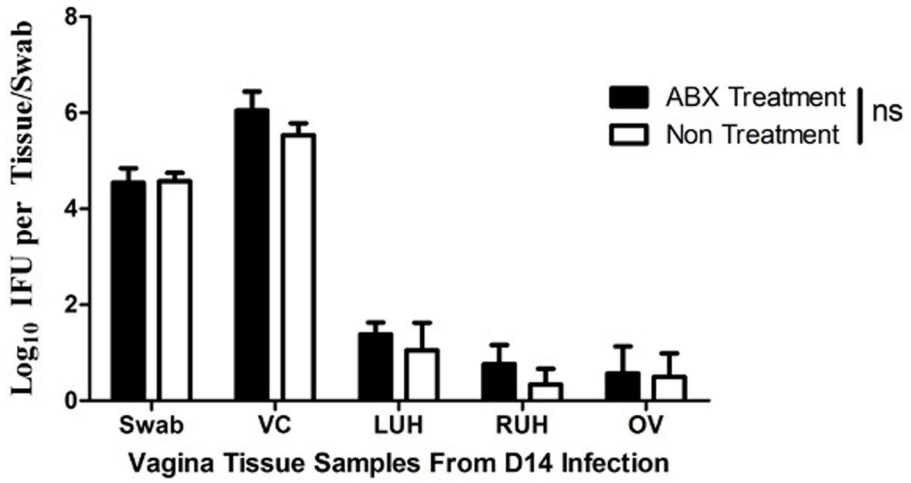

FIGURE 3
Oral antibiotics treatment fails to alter *C. muridarum* ascension to the upper genital tract. Female DBA2/J mice without (Non, *n*=5) or with (ABX, *n*=5) oral antibiotics treatment for 21days were intravaginally inoculated with *C. muridarum*. The oral ABX was maintained in drinking water throughout the experiment. On day 14 after the intravaginal inoculation, all mice were sacrificed for titrating live organisms recovered from different regions of the genital tract including vagina/cervix (VC), left uterine horn (LU), right uterine horn (RU) and oviduct/ovary (RO) as shown along X-axis. The live organisms recovered were expressed as Log10 IFUs as shown along *Y*-axis. Note that live *C. muridarum* organisms were similarly detected in uterine horn and oviduct tissues of all mice regardless of the oral antibiotics' treatment ($p>0.05$, Wilcoxon for comparing IFUs from individual tissues between the two groups of mice).

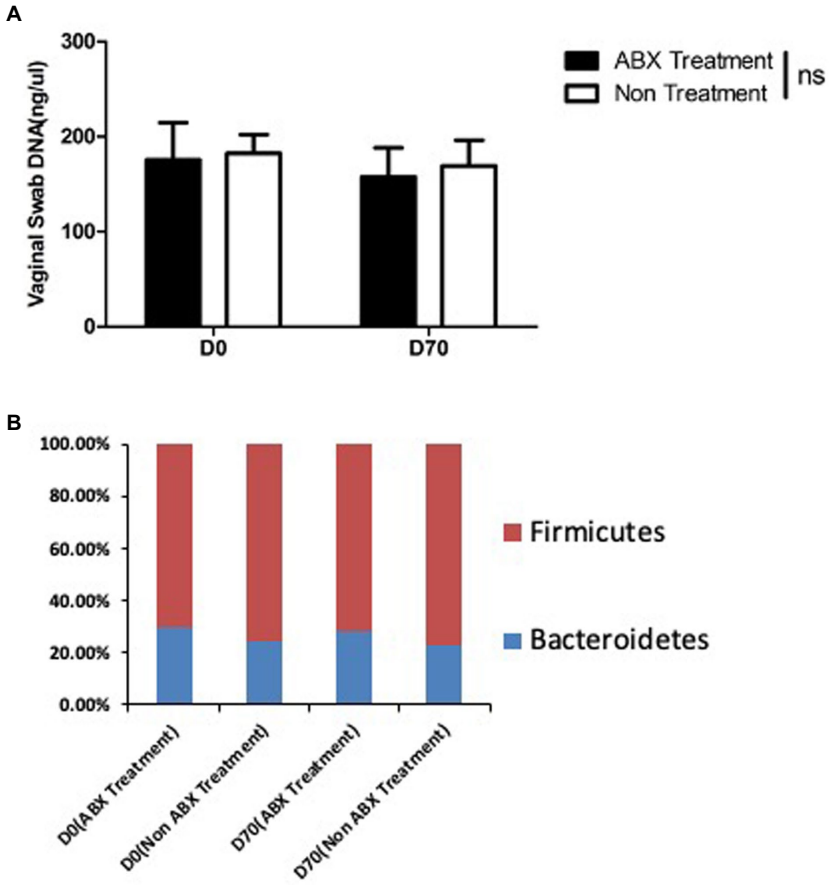

FIGURE 4
Oral antibiotics treatment fails to alter vaginal microbiota. Female DBA2/J mice were treated without (Non, *n*=20) or with (ABX, *n*=20) oral antibiotics for 21days (D0 or prior to infection) or 91days (D70, 70days after infection) as described in Figure 1 legend. Vaginal swabs were taken for extracting DNA (panel **A**) and detecting the relative abundance of 16S rRNA gene from bacterial phyla bacteroidetes (blue, panel **B**) and firmicutes (red, panel **B**) using. Neither the vaginal DNA amount nor the relative abundance of bacteroidetes and firmicutes in the vaginal swabs was different between mice with or without the oral antibiotics treatment for 21 or 91days ($p>0.05$, Wilcoxon).

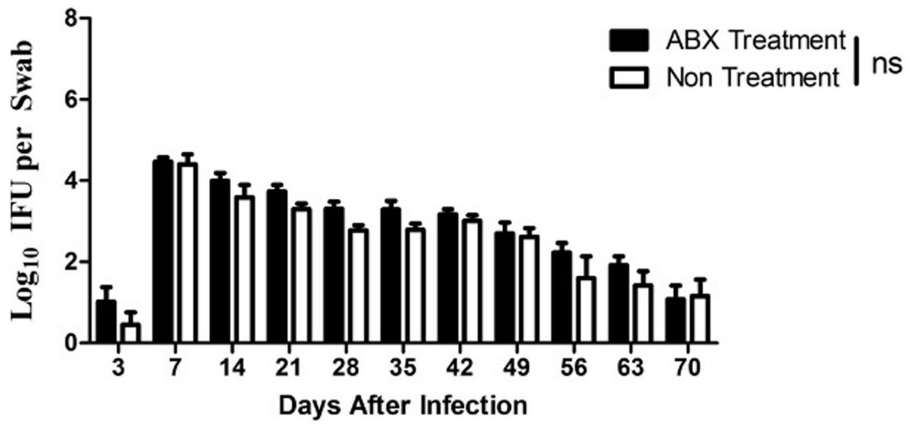

FIGURE 5
Oral antibiotics treatment did not alter the *C. muridarum* colonization in the gut. Female DBA2/J mice without (Non, *n*=20) or with (ABX, *n*=29) oral antibiotics treatment was intravaginally infected with *C. muridarum* as described in Figure 1 legend. Live organism shedding was monitored from rectal swabs on days 3, 7 and weekly thereafter as shown along *X*-axis. The live organisms recovered (Log10 IFUs per swab) were shown along Y axis. Note that significant shedding was similarly detected in all mice throughout the experiment regardless of the oral antibiotics treatment ($p>0.05$, Wilcoxon for comparing area under curve).

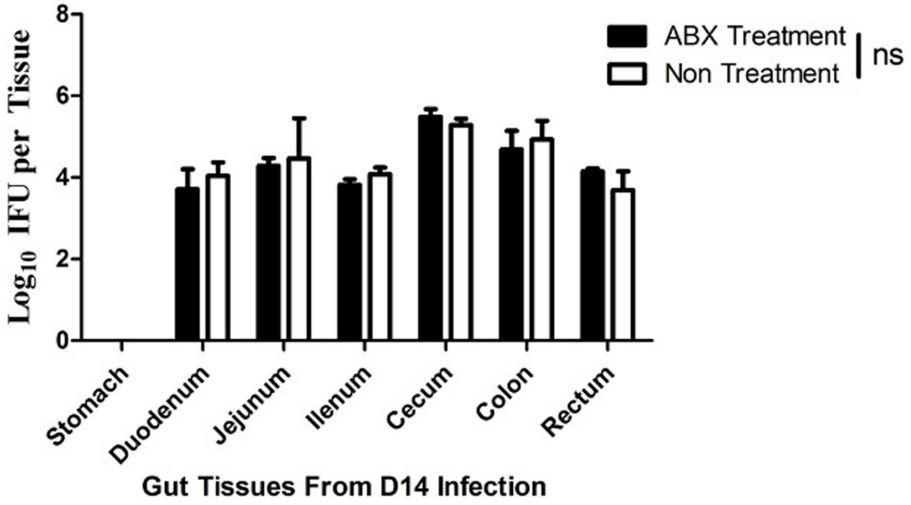

FIGURE 6
Oral antibiotics treatment fails to alter *C. muridarum* distribution in the mouse gastrointestinal tract. Female DBA2/J mice without (Non, *n*=5) or with (ABX) oral antibiotics treatment for 21days were intravaginally inoculated with *C. muridarum* as described in Figure 3 legend. On day 14 after the intravaginal inoculation, all mice were sacrificed for titrating live organisms recovered from different regions of the gastrointestinal tract including stomach, duodenum, jejunum, ilium, cecum, colon and rectum as shown along *X*-axis. The live organisms recovered were expressed as Log10 IFUs as shown along *Y*-axis. Note that live *C. muridarum* organisms were similarly detected in different regions of the gastrointestinal tracts from all mice regardless of the oral antibiotics treatment ($p>0.05$, Wilcoxon for comparing IFUs from individual tissues between the two groups of mice).

We found that 21 days of ABX treatment in mice result in significant dysbiosis. The dysbiosis was maintained till the end of the experiments (up to 70 days after infection). The total fecal DNA of oral ABX-treated mice was significantly reduced, suggesting that the oral ABX treatment significantly reduced the number of bacteria in the gut. The reduced amount of fecal DNA reflected the reduction in the bacteria burden, while whether the bacterial composition or diversity has been changed is not clear. We then monitored the composition of bacterial phyla and we found significant alteration of bacterial composition with decreased bacteroidetes but increased firmicutes.

Oral ABX-related hydrosalpinx correlates with GI microbiota profile

The oral ABX treatment promoted hydrosalpinx development and induced gut dysbiosis without altering microbiota in the genital tract. The GI and genital chlamydial colonization wasn't influenced by ABX treatment either. We used a Spearman's correlation analysis to mathematically define the correlation (Figure 8) between the hydrosalpinx scores and gut dysbiosis. It is shown that the hydrosalpinx scores positively correlated with gut dysbiosis scores expressed as Log10 F/B ratios obtained from feces. However, the hydrosalpinx scores did not

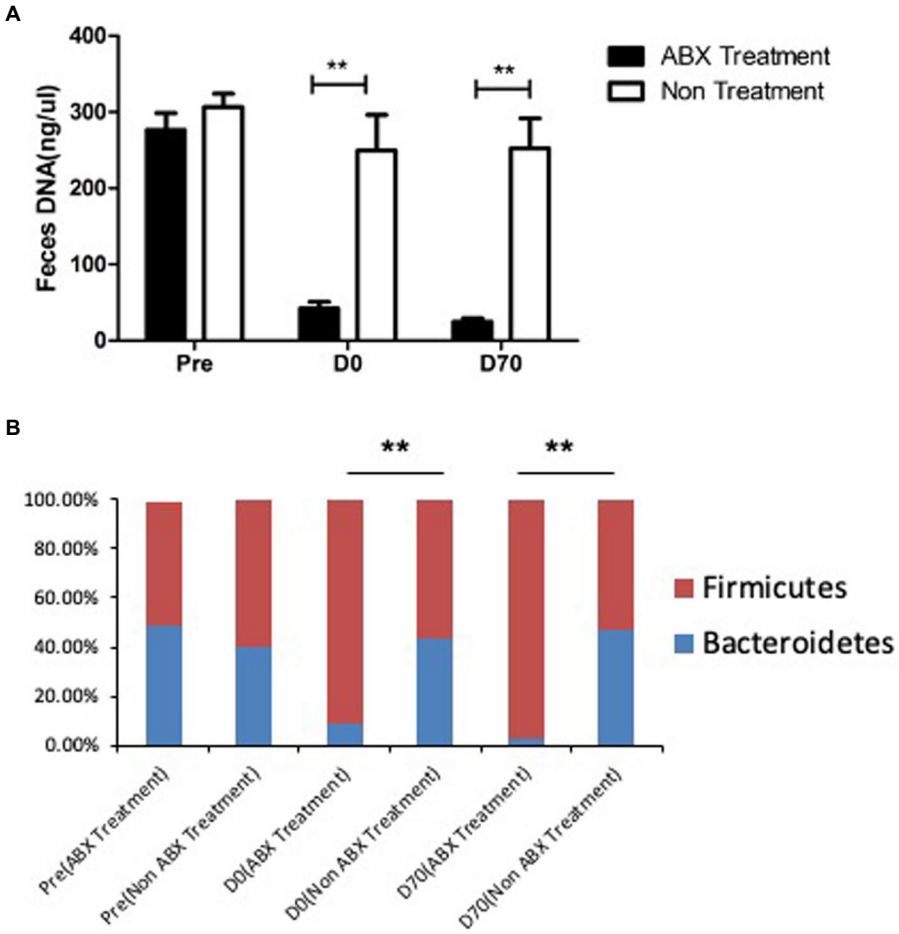

FIGURE 7
The fecal microbiota were changed sigificantly by oral antibiotics treatment. Female DBA2/J mice were treated without (Non, n=20) or with (ABX, n=20) oral antibiotics for 21days as described in Figure 1 legend. Fecal samples were taken from mice prior to the oral antibiotics treatment (Pre), 21days after the treatment but before chlamydial infection (D0) and 70days after chlamydial infection (D70) for extracting DNA (panel **A**) and detecting the relative abundance of 16S rRNA gene from bacterial phyla bacteroidetes (blue, panel **B**) and firmicutes (red, panel **B**) using qPCR. Please note that both fecal DNA amount and relative abundance of bacteroidetes and firmicutes in the fecal samples were significantly different between mice with or without the oral antibiotics treatment for 21days (**p<0.01, Wilcoxon).

correlate with Log10 F/B ratios from vaginal swabs or live chlamydial organism shedding (Log10 IFUs) from rectal or vaginal swabs.

The correlation co-efficient between the hydrosalpinx scores and gut dysbiosis was 0.51 (with a p value of 0.0039) and the correlation co-efficient between the hydrosalpinx scores and vaginal F/B ratios or rectal IFUs vaginal IFUs were −0.0416 (p = 0.89), 0.0048 (p = 0.9763), and 0.1094 (p = 0.5665) respectively (Figure 8). The significant mathematical correlation was only identified between the hydrosalpinx scores and fecal F/B ratios. This correlation was significantly stronger than those between hydrosalpinx and vaginal F/B ratio or rectal or vaginal IFUs (p = 0.0455, 0.0183, 0.0475 respectively). As a result, we identified a significant association of gut dysbiosis with chlamydial pathogenicity in the upper genital tract.

Discussion

In the current study, we used an oral ABX cocktail to induce gut dysbiosis in the DBA2/J mice with *C. muridarum* genital infection and we tried to demonstrate the role of gut dysbiosis in chlamydial pathogenicity in the upper genital tract with this model. The oral administration of membrane impermeable antibiotics vancomycin and gentamicin significantly enhanced hydrosalpinx development in DBA2/J mice with *C. muridarum* genital infection. The DBA2/J mice were known to be highly resistant to hydrosalpinx induction by gential *C. muridarum* infection, while the oral ABX treatment consistently enhanced hydrosalpinx development. This enhancement effect was demonstrated in 3 independent experiments. Firstly, the ABX treatment did not significantly alter either chlamydial infection in the genital tract, and it suggested that the oral ABX promoted hydrosalpinx through the mechanisms independent of the genital Chlamydia. Furthermore, the oral ABX treatment did not affect genital *C. muridarum* spreading to or colonizing in the GI tract, suggesting that the oral ABX treatment did not promote hydrosalpinx by increasing chlamydial colonization in GI tract either. Finally, the oral ABX caused severe dysbiosis in the GI tract, including reduction in fecal DNA and increase in ratios of firmicutes over bacteroidetes. The oral antibiotics- enhanced hydrosalpinx only correlated with

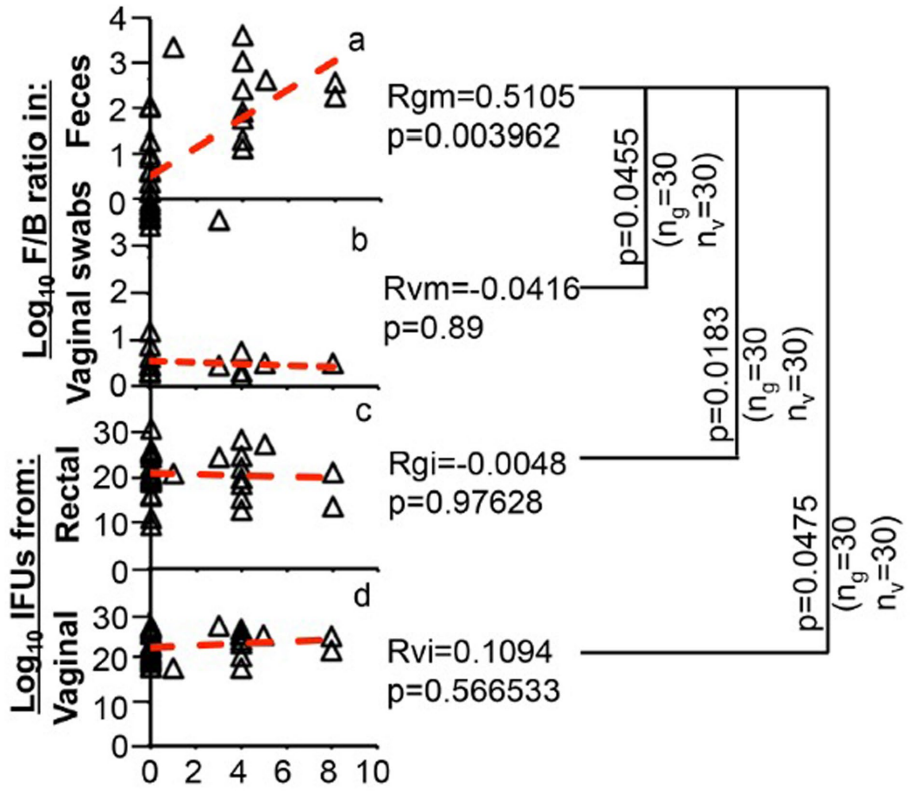

FIGURE 8
Correlation of oral antibiotics-enhanced hydrosalpinx with fecal microbiota but not vaginal microbiota or *C. muridarum* shedding from either genital or gastrointestinal tract. The hydrosalpinx score from each DBA2/J mouse as described in Figure 1 legend (*X*-axis) was used to correlate with microbiota profile expressed as Log10 F/B ratios obtained from feces (*Y*-axis, panel a) or vaginal swabs (*Y*-axis, b) or live organism shedding expressed as Log10 IFUs (total IFU from swabs collected from a given mouse over time, *Y*-axis) from rectal (c) or vaginal (d) swabs using the Spearman's correlation formula as described in the materials and method section. The total IFU from each mouse was obtained by adding IFUs from all time points observed. The correlation co-efficient between the hydrosalpinx scores and gut (or feces) F/B ratios was defined as Rgm, which is 0.5105 with a *p* value of 0.0039, the correlation co-efficient between the hydrosalpinx scores and vaginal F/B ratios was Rvm=−0.0416 with a $p=0.89$ (two tails), or rectal IFUs (Rgi=0.0048, $p=0.9763$) or vaginal IFUs (Rvi=0.1094, $p=0.5665$). A positive correlation was only identified between the hydrosalpinx scores and rectal F/B ratios, which was significantly stronger than those between hydrosalpinx and rectal F/B ratio or rectal or vaginal IFUs ($p=0.0455$, 0.0183, 0.0475 respectively).

dysbiosis in the gut but not genital tract. It is noteworthy to emphasize that although the Firmicutes to Bacteroidetes ratio in the gut has been considered as an indicator of dysbiosis (Stojanov et al., 2020; Kapoor et al., 2023), this metric alone may not sufficiently discriminate differences in the bacterial species composition. In addition, it may not provide insights into the changes in bacterial diversity and evenness, or the co-occurrence of bacterial networks associated with specific microbiota states as potential pathological biomarkers. Therefore, more advanced metagenomic approaches (Gupta et al., 2023; Szóstak et al., 2023), such as 16S rRNA sequencing or shotgun sequencing of all bacterial genomes, may be required to explicitly address these issues. Despite the limitations, our study provides a new direction for further understanding chlamydial pathogenicity.

The oral antibiotics used in many other gut microbiome studies often include neomycin, ampicillin and metronidazole in addiction to vancomycin or gentamicin (Kennedy et al., 2018). In order to selectively target gut lumenal bacteria, we used a cocktail consisting of only vancomycin and gentamicin since these two antibiotics cannot pass through gut epithelial barrier and fail to inhibit chlamydial growth (Ridgway et al., 1978; Dailloux et al., 1990). Indeed, we observed that the orally delivered vancomycin and gentamicin cocktail did not alter colonization of *C. muridarum* nor other bacteria in the genital tract. It failed to affect genital *C. muridarum* spreading to and colonizing in the GI tract as well. This selective targeting of gut lumenal bacteria by the oral ABX used in the current study has allowed us to associate the antibiotics-enhanced hydrosalpinx with gut dysbiosis. The next question is how the oral ABX-induced gut dysbiosis enhanced chlamydial pathogenicity in the upper genital tract. In recent years, gut microbiota has been associated with extra-gut pathologies (Tai et al., 2016; Shivaji, 2017). It is suggested that gut microbiota may even regulate fibrosis (Burke et al., 2017; Ho and Varga, 2017; Loomba et al., 2017). Since fibrosis is a key feature of chlamydial pathogenicity in the upper genital tract (Rodgers et al., 2010, 2011; Budrys et al., 2012; Chen et al., 2014; Lei et al., 2014; Liu et al., 2014; Yang et al., 2014; Zhong, 2017), we hypothesize that gut dysbiosis induced by the oral ABX in DBA2/J mice may promote profibrotic responses in the genital tract following chlamydial infection.

Normal microbiota is required for maintaining immunity to insults (Belkaid and Hand, 2014). Gut dysbiosis may skew the host immune responses toward Th2-dominant response that may favor

tissue repairing and fibrosis. The increase in the ratio of Firmicutes over Bacteroidetes in gut microbiota has been associated with exacerbation of pathologic responses (Chen et al., 2011; Russell et al., 2012; Arrieta et al., 2014; Belkaid and Hand, 2014; Arrieta et al., 2015; Block et al., 2016; Brown et al., 2017; Budden et al., 2017; Ipci et al., 2017; Kang et al., 2017; Lin and Zhang, 2017; Samuelson et al., 2017; Shivaji, 2017; Wood et al., 2017). The increased ratio of Firmicutes over Bacteroidetes in gut microbiota can also be accompanied with increased segmental filamentous bacteria (SFB) that are known to induce Th17 (Gauguet et al., 2015; McAleer et al., 2016). Although Th17 is not as effective as Th1 in clearing chlamydial infection, Th17 cells have been shown to promote chlamydial pathogenicity in the upper genital tract (Moore-Connors et al., 2013). Thus, we assume that the oral ABX-induced dysbiosis may promote chlamydial induction of hydrosalpinx without significantly altering chlamydial infectivity. We will use the oral vancomycin and gentamicin treatment of DBA2/J mouse intravaginal infection model to testing this and other hypotheses in future studies.

Limitations of the study

The current study has a limitation in that it utilized real-time quantitative PCR to target only total 16S rRNA belonging exclusively to the bacterial phyla Bacteroidetes and Firmicutes to investigate the gut and genital microbiota. The approach is inadequate for assessing bacterial taxonomic diversity and track shifts in bacterial community structure and organization, which could have diagnostic value as indicators of pathological conditions. More advanced metagenomic approaches, such as 16S rRNA sequencing or shotgun sequencing of all bacterial genomes, were not utilized in this study and are required in order to perform such analyses.

Additionally, the study solely focused on DBA/2J mice with oral antibiotic-induced dysbiosis as a model for investigating the association between gut microbiota and chlamydial genital pathogenicity. It remains unknown if these findings can be extrapolated to other chlamydia-resistant mice models.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

All of the animal studies were approved by the Ethics Committee of the Institute of Pasteur of Shanghai Chinese Academy of Sciences.

Author contributions

QT, TZ, and LW: conceptualization. QT, TZ, JM, and XS: methodology. QT, TZ, LW, and XS: investigation. QT, TZ, and LW: writing—original draft. QT and XS: writing—review and editing. TZ and QT: supervision. TZ and QT: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

References

Andrew, D. W., Cochrane, M., Schripsema, J. H., Ramsey, K. H., Dando, S. J., O'Meara, C. P., et al. (2013). The duration of *Chlamydia muridarum* genital tract infection and associated chronic pathological changes are reduced in IL-17 knockout mice but protection is not increased further by immunization. *PLoS One* 8:e76664. doi: 10.1371/journal.pone.0076664

Arrieta, M. C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M., and Finlay, B. (2014). The intestinal microbiome in early life: health and disease. *Front. Immunol.* 5:427. doi: 10.3389/fimmu.2014.00427

Arrieta, M. C., Stiemsma, L. T., Dimitriu, P. A., Thorson, L., Russell, S., Yurist-Doutsch, S., et al. (2015). Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci. Transl. Med.* 7:307ra152. doi: 10.1126/scitranslmed.aab2271

Asquith, K. L., Horvat, J. C., Kaiko, G. E., Carey, A. J., Beagley, K. W., Hansbro, P. M., et al. (2011). Interleukin-13 promotes susceptibility to chlamydial infection of the respiratory and genital tracts. *PLoS Pathog.* 7:e1001339. doi: 10.1371/journal.ppat.1001339

Bacchetti De Gregoris, T., Aldred, N., Clare, A. S., and Burgess, J. G. (2011). Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *J. Microbiol. Methods* 86, 351–356. doi: 10.1016/j.mimet.2011.06.010

Bai, G., Gajer, P., Nandy, M., Ma, B., Yang, H., Sakamoto, J., et al. (2012). Comparison of storage conditions for human vaginal microbiome studies. *PLoS One* 7:e36934. doi: 10.1371/journal.pone.0036934

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cells* 157, 121–141. doi: 10.1016/j.cell.2014.03.011

Block, K. E., Zheng, Z., Dent, A. L., Kee, B. L., and Huang, H. (2016). Gut microbiota regulates K/BxN autoimmune arthritis through follicular helper T but not Th17 cells. *J. Immunol.* 196, 1550–1557. doi: 10.4049/jimmunol.1501904

Brown, R. L., Sequeira, R. P., and Clarke, T. B. (2017). The microbiota protects against respiratory infection via GM-CSF signaling. *Nat. Commun.* 8:1512. doi: 10.1038/s41467-017-01803-x

Budden, K. F., Gellatly, S. L., Wood, D. L., Cooper, M. A., Morrison, M., Hugenholtz, P., et al. (2017). Emerging pathogenic links between microbiota and the gut-lung axis. *Nat. Rev. Microbiol.* 15, 55–63. doi: 10.1038/nrmicro.2016.142

Budrys, N. M., Gong, S., Rodgers, A. K., Wang, J., Louden, C., Shain, R., et al. (2012). Chlamydia trachomatis antigens recognized in women with tubal factor infertility, normal fertility, and acute infection. *Obstet. Gynecol.* 119, 1009–1016. doi: 10.1097/AOG.0b013e3182519326

Burke, D. G., Fouhy, F., Harrison, M. J., Rea, M. C., Cotter, P. D., O'Sullivan, O., et al. (2017). The altered gut microbiota in adults with cystic fibrosis. *BMC Microbiol.* 17:58. doi: 10.1186/s12866-017-0968-8

Chen, L. W., Chen, P. H., and Hsu, C. M. (2011). Commensal microflora contribute to host defense against Escherichia coli pneumonia through toll-like receptors. *Shock* 36, 67–75. doi: 10.1097/SHK.0b013e3182184ee7

Chen, L., Cheng, W., Shivshankar, P., Lei, L., Zhang, X., Wu, Y., et al. (2009). Distinct roles of CD28- and CD40 ligand-mediated costimulation in the development of protective immunity and pathology during *Chlamydia muridarum* urogenital infection in mice. *Infect. Immun.* 77, 3080–3089. doi: 10.1128/IAI.00611-08

Chen, L., Lei, L., Chang, X., Li, Z., Lu, C., Zhang, X., et al. (2010). Mice deficient in MyD88 develop a Th2-dominant response and severe pathology in the upper genital tract following *Chlamydia muridarum* infection. *J. Immunol.* 184, 2602–2610. doi: 10.4049/jimmunol.0901593

Chen, J., Zhang, H., Zhou, Z., Yang, Z., Ding, Y., Zhou, Z., et al. (2014). Chlamydial induction of hydrosalpinx in 11 strains of mice reveals multiple host mechanisms for preventing upper genital tract pathology. *PLoS One* 9:e95076. doi: 10.1371/journal.pone.0095076

Chen, C., Zhou, Z., Conrad, T., Yang, Z., Dai, J., Li, Z., et al. (2015). In vitro passage selects for *Chlamydia muridarum* with enhanced infectivity in cultured cells but attenuated pathogenicity in mouse upper genital tract. *Infect. Immun.* 83, 1881–1892. doi: 10.1128/IAI.03158-14

Cheng, W., Shivshankar, P., Li, Z., Chen, L., Yeh, I. T., and Zhong, G. (2008). Caspase-1 contributes to chlamydia trachomatis-induced upper urogenital tract inflammatory pathologies without affecting the course of infection. *Infect. Immun.* 76, 515–522. doi: 10.1128/IAI.01064-07

Dailloux, M., Ottemer, C., Albert, H., and Weber, M. (1990). In vitro sensitivity of Chlamydiae to antibiotics. *Pathol Biol (Paris)* 38, 426–430.

Dong, X., Liu, Y., Chang, X., Lei, L., and Zhong, G. (2014). Signaling via tumor necrosis factor receptor 1 but not toll-like receptor 2 contributes significantly to hydrosalpinx development following *Chlamydia muridarum* infection. *Infect. Immun.* 82, 1833–1839. doi: 10.1128/IAI.01668-13

Fan, T., Lu, H., Hu, H., Shi, L., McClarty, G. A., Nance, D. M., et al. (1998). Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *J. Exp. Med.* 187, 487–496. doi: 10.1084/jem.187.4.487

Frazer, L. C., Sullivan, J. E., Zurenski, M. A., Mintus, M., Tomasak, T. E., Prantner, D., et al. (2013). CD4+ T cell expression of MyD88 is essential for normal resolution of *Chlamydia muridarum* genital tract infection. *J. Immunol.* 191, 4269–4279. doi: 10.4049/jimmunol.1301547

Gauguet, S., D'Ortona, S., Ahnger-Pier, K., Duan, B., Surana, N. K., Lu, R., et al. (2015). Intestinal microbiota of mice influences resistance to Staphylococcus aureus pneumonia. *Infect. Immun.* 83, 4003–4014. doi: 10.1128/IAI.00037-15

Gupta, Y., Ernst, A. L., Vorobyev, A., Beltsiou, F., Zillikens, D., Bieber, K., et al. (2023). Impact of diet and host genetics on the murine intestinal mycobiome. *Nat. Commun.* 14:834. doi: 10.1038/s41467-023-36479-z

Hickey, R. J., Zhou, X., Pierson, J. D., Ravel, J., and Forney, L. J. (2012). Understanding vaginal microbiome complexity from an ecological perspective. *Transl. Res.* 160, 267–282. doi: 10.1016/j.trsl.2012.02.008

Ho, K. J., and Varga, J. (2017). Early-life gut Dysbiosis: a driver of later-life fibrosis? *J. Invest. Dermatol.* 137, 2253–2255. doi: 10.1016/j.jid.2017.08.017

Huang, Y., Zhang, Q., Yang, Z., Conrad, T., Liu, Y., and Zhong, G. (2015). Plasmid-encoded Pgp5 is a significant contributor to *Chlamydia muridarum* induction of Hydrosalpinx. *PLoS One* 10:e0124840. doi: 10.1371/journal.pone.0124840

Ipci, K., Altintoprak, N., Muluk, N. B., Senturk, M., and Cingi, C. (2017). The possible mechanisms of the human microbiome in allergic diseases. *Eur. Arch. Otorhinolaryngol.* 274, 617–626. doi: 10.1007/s00405-016-4058-6

Jansen, K., Schmidt, A. J., Drewes, J., Bremer, V., and Marcus, U. (2016). Increased incidence of syphilis in men who have sex with men and risk management strategies, Germany, 2015. *Euro Surveill.* 21:30382. doi: 10.2807/1560-7917.ES.2016.21.43.30382

Kang, Y. B., Cai, Y., and Zhang, H. (2017). Gut microbiota and allergy/asthma: from pathogenesis to new therapeutic strategies. *Allergol. Immunopathol.* 45, 305–309. doi: 10.1016/j.aller.2016.08.004

Kapoor, P., Tiwari, A., Sharma, S., Tiwari, V., Sheoran, B., Ali, U., et al. (2023). Effect of anthocyanins on gut health markers, Firmicutes-Bacteroidetes ratio and short-chain fatty acids: a systematic review via meta-analysis. *Sci. Rep.* 13:1729. doi: 10.1038/s41598-023-28764-0

Kennedy, E. A., King, K. Y., and Baldridge, M. T. (2018). Mouse microbiota models: comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Front. Physiol.* 9:1534. doi: 10.3389/fphys.2018.01534

Koch, K. N., and Muller, A. (2015). Helicobacter pylori activates the TLR2/NLRP3/caspase-1/IL-18 axis to induce regulatory T-cells, establish persistent infection and promote tolerance to allergens. *Gut Microbes* 6, 382–387. doi: 10.1080/19490976.2015.1105427

Land, J. A., van Bergen, J. E. A. M., Morre, S. A., and Postma, M. J. (2010). Epidemiology of chlamydia trachomatis infection in women and the cost-effectiveness of screening. *Hum. Reprod. Update* 16, 189–204. doi: 10.1093/humupd/dmp035

Lei, L., Chen, J., Hou, S., Ding, Y., Yang, Z., Zeng, H., et al. (2014). Reduced live organism recovery and lack of hydrosalpinx in mice infected with plasmid-free *Chlamydia muridarum*. *Infect. Immun.* 82, 983–992. doi: 10.1128/IAI.01543-13

Li, Z., Lu, C., Peng, B., Zeng, H., Zhou, Z., Wu, Y., et al. (2012). Induction of protective immunity against *Chlamydia muridarum* intravaginal infection with a chlamydial glycogen phosphorylase. *PLoS One* 7:e32997. doi: 10.1371/journal.pone.0032997

Li, C. W., Xia, W., Huo, L., Lim, S. O., Wu, Y., Hsu, J. L., et al. (2012). Epithelial-mesenchymal transition induced by TNF-alpha requires NF-kappaB-mediated transcriptional upregulation of Twist1. *Cancer Res.* 72, 1290–1300. doi: 10.1158/0008-5472.CAN-11-3123

Lin, L., and Zhang, J. (2017). Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunol.* 18:2. doi: 10.1186/s12865-016-0187-3

Liu, Y., Huang, Y., Yang, Z., Sun, Y., Gong, S., Hou, S., et al. (2014). Plasmid-encoded Pgp3 is a major virulence factor for *Chlamydia muridarum* to induce hydrosalpinx in mice. *Infect. Immun.* 82, 5327–5335. doi: 10.1128/IAI.02576-14

Loomba, R., Seguritan, V., Li, W., Long, T., Klitgord, N., Bhatt, A., et al. (2017). Gut microbiome-based metagenomic signature for non-invasive detection of advanced fibrosis in human nonalcoholic fatty liver disease. *Cell Metab.* 30:e1055. doi: 10.1016/j.cmet.2019.08.002

Lu, C., Zeng, H., Li, Z., Lei, L., Yeh, I. T., Wu, Y., et al. (2012). Protective immunity against mouse upper genital tract pathology correlates with high IFNγbut low IL-17 T cell and anti-secretion protein antibody responses induced by replicating chlamydial organisms in the airway. *Vaccine* 30, 475–485. doi: 10.1016/j.vaccine.2011.10.059

Ma, B., Forney, L. J., and Ravel, J. (2012). Vaginal microbiome: rethinking health and disease. *Annu. Rev. Microbiol.* 66, 371–389. doi: 10.1146/annurev-micro-092611-150157

Manam, S., Thomas, J. D., Li, W., Maladore, A., Schripsema, J. H., Ramsey, K. H., et al. (2015). Tumor necrosis factor (TNF) receptor superfamily member 1b on CD8+ T cells and TNF receptor superfamily member 1a on non-CD8+ T cells contribute significantly to upper genital tract pathology following chlamydial infection. *J. Infect. Dis.* 211, 2014–2022. doi: 10.1093/infdis/jiu839

McAleer, J. P., Nguyen, N. L., Chen, K., Kumar, P., Ricks, D. M., Binnie, M., et al. (2016). Pulmonary Th17 antifungal immunity is regulated by the gut microbiome. *J. Immunol.* 197, 97–107. doi: 10.4049/jimmunol.1502566

Metwali, A., Setiawan, T., Blum, A. M., Urban, J., Elliott, D. E., Hang, L., et al. (2006). Induction of CD8+ regulatory T cells in the intestine by Heligmosomoides polygyrus infection. *Am. J. Physiol. Gastrointest. Liver Physiol.* 291, G253–G259. doi: 10.1152/ajpgi.00409.2005

Moore-Connors, J. M., Fraser, R., Halperin, S. A., and Wang, J. (2013). CD4(+) CD25(+)Foxp3(+) regulatory T cells promote Th17 responses and genital tract inflammation upon intracellular *Chlamydia muridarum* infection. *J. Immunol.* 191, 3430–3439. doi: 10.4049/jimmunol.1301136

Murthy, A. K., Chambers, J. P., Meier, P. A., Zhong, G., and Arulanandam, B. P. (2007). Intranasal vaccination with a secreted chlamydial protein enhances resolution of genital *Chlamydia muridarum* infection, protects against oviduct pathology, and is highly dependent upon endogenous gamma interferon production. *Infect. Immun.* 75, 666–676. doi: 10.1128/IAI.01280-06

Murthy, A. K., Li, W., Chaganty, B. K., Kamalakaran, S., Guentzel, M. N., Seshu, J., et al. (2011). Tumor necrosis factor alpha production from CD8+ T cells mediates oviduct pathological sequelae following primary genital *Chlamydia muridarum* infection. *Infect. Immun.* 79, 2928–2935. doi: 10.1128/IAI.05022-11

Nardini, P., Ñahui Palomino, R. A., Parolin, C., Laghi, L., Foschi, C., Cevenini, R., et al. (2016). Lactobacillus crispatus inhibits the infectivity of chlamydia trachomatis elementary bodies, in vitro study. *Sci. Rep.* 6:29024. doi: 10.1038/srep29024

Natividad, A., Hanchard, N., Holland, M. J., Mahdi, O. S., Diakite, M., Rockett, K., et al. (2007). Genetic variation at the TNF locus and the risk of severe sequelae of ocular chlamydia trachomatis infection in Gambians. *Genes Immun.* 8, 288–295. doi: 10.1038/sj.gene.6364384

Nelson, D. E., van der Pol, B., Dong, Q., Revanna, K. V., Fan, B., Easwaran, S., et al. (2010). Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. *PLoS One* 5:e14116. doi: 10.1371/journal.pone.0014116

Noyes, N., Cho, K. C., Ravel, J., Forney, L. J., and Abdo, Z. (2018). Associations between sexual habits, menstrual hygiene practices, demographics and the vaginal

microbiome as revealed by Bayesian network analysis. *PLoS One* 13:e0191625. doi: 10.1371/journal.pone.0191625

Owyang, A. M., Zaph, C., Wilson, E. H., Guild, K. J., McClanahan, T., Miller, H. R., et al. (2006). Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J. Exp. Med.* 203, 843–849. doi: 10.1084/jem.20051496

Peng, B., Lu, C., Tang, L., Yeh, I. T., He, Z., Wu, Y., et al. (2011). Enhanced upper genital tract pathologies by blocking Tim-3 and PD-L1 signaling pathways in mice intravaginally infected with *Chlamydia muridarum*. *BMC Infect. Dis.* 11:347. doi: 10.1186/1471-2334-11-347

Ravel, J., and Brotman, R. M. (2016). Translating the vaginal microbiome: gaps and challenges. *Genome Med.* 8:35. doi: 10.1186/s13073-016-0291-2

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U. S. A.* 108, 4680–4687. doi: 10.1073/pnas.1002611107

Ridgway, G. L., Owen, J. M., and Oriel, J. D. (1978). The antimicrobial susceptibility of chlamydia trachomatis in cell culture. *Br. J. Vener. Dis.* 54, 103–106. doi: 10.1136/sti.54.2.103

Rodgers, A. K., Budrys, N. M., Gong, S., Wang, J., Holden, A., Schenken, R. S., et al. (2011). Genome-wide identification of chlamydia trachomatis antigens associated with tubal factor infertility. *Fertil. Steril.* 96, 715–721. doi: 10.1016/j.fertnstert.2011.06.021

Rodgers, A. K., Wang, J., Zhang, Y., Holden, A., Berryhill, B., Budrys, N. M., et al. (2010). Association of tubal factor infertility with elevated antibodies to chlamydia trachomatis caseinolytic protease P. *Am. J. Obstet. Gynecol.* 203, e497–5 e414. doi: 10.1016/j.ajog.2010.06.005

Russell, S. L., Gold, M. J., Hartmann, M., Willing, B. P., Thorson, L., Wlodarska, M., et al. (2012). Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep.* 13, 440–447. doi: 10.1038/embor.2012.32

Samuelson, D. R., Shellito, J. E., Maffei, V. J., Tague, E. D., Campagna, S. R., Blanchard, E. E., et al. (2017). Alcohol-associated intestinal dysbiosis impairs pulmonary host defense against Klebsiella pneumoniae. *PLoS Pathog.* 13:e1006426. doi: 10.1371/journal.ppat.1006426

Shivaji, S. (2017). We are not alone: a case for the human microbiome in extra intestinal diseases. *Gut Pathog.* 9:13. doi: 10.1186/s13099-017-0163-3

Stojanov, S., Berlec, A., and Štrukelj, B. (2020). The influence of probiotics on the Firmicutes/Bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. *Microorganisms* 8:1715. doi: 10.3390/microorganisms8111715

Strandell, A., Waldenstrom, U., Nilsson, L., and Hamberger, L. (1994). Hydrosalpinx reduces in-vitro fertilization/embryo transfer pregnancy rates. *Hum. Reprod.* 9, 861–863. doi: 10.1093/oxfordjournals.humrep.a138606

Szóstak, N., Handschuh, L., Samelak-Czajka, A., Tomela, K., Schmidt, M., Pruss, Ł., et al. (2023). Host factors associated with gut Mycobiome structure. *mSystems*:e0098622. doi: 10.1128/msystems.00986-22

Tai, N., Peng, J., Liu, F., Gulden, E., Hu, Y., Zhang, X., et al. (2016). Microbial antigen mimics activate diabetogenic CD8 T cells in NOD mice. *J. Exp. Med.* 213, 2129–2146. doi: 10.1084/jem.20160526

Tang, L., Yang, Z., Zhang, H., Zhou, Z., Arulanandam, B., Baseman, J., et al. (2014). Induction of protective immunity against *Chlamydia muridarum* intracervical infection in DBA/1j mice. *Vaccine* 32, 1407–1413. doi: 10.1016/j.vaccine.2013.10.018

van Houdt, R., Ma, B., Bruisten, S. M., Speksnijder, A., Ravel, J., and de Vries, H. J. C. (2018). Lactobacillus iners-dominated vaginal microbiota is associated with increased susceptibility to chlamydia trachomatis infection in Dutch women: a case-control study. *Sex. Transm. Infect.* 94, 117–123. doi: 10.1136/sextrans-2017-053133

Vasquez, G., Boeckx, W., and Brosens, I. (1995). Prospective study of tubal mucosal lesions and fertility in hydrosalpinges. *Hum. Reprod.* 10, 1075–1078. doi: 10.1093/oxfordjournals.humrep.a136097

Wood, M. R., Yu, E. A., and Mehta, S. (2017). The human microbiome in the fight against tuberculosis. *Am. J. Trop. Med. Hyg.* 96, 1274–1284. doi: 10.4269/ajtmh.16-0581

Yadav, S. K., Boppana, S., Ito, N., Mindur, J. E., Mathay, M. T., Patel, A., et al. (2017). Gut dysbiosis breaks immunological tolerance toward the central nervous system during young adulthood. *Proc. Natl. Acad. Sci. U. S. A.* 114, E9318–E9327. doi: 10.1073/pnas.1615715114

Yang, Y. W., Chen, M. K., Yang, B. Y., Huang, X. J., Zhang, X. R., He, L. Q., et al. (2015). Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in mouse feces. *Appl. Environ. Microbiol.* 81, 6749–6756. doi: 10.1128/AEM.01906-15

Yang, Z., Conrad, T., Zhou, Z., Chen, J., Dutow, P., Klos, A., et al. (2014). Complement factor C5 but not C3 contributes significantly to hydrosalpinx development in mice infected with *Chlamydia muridarum*. *Infect. Immun.* 82, 3154–3163. doi: 10.1128/IAI.01833-14

Zhang, Q., Huang, Y., Gong, S., Yang, Z., Sun, X., Schenken, R., et al. (2015). In vivo and ex vivo imaging reveals a Long-lasting chlamydial infection in the mouse gastrointestinal tract following genital tract inoculation. *Infect. Immun.* 83, 3568–3577. doi: 10.1128/IAI.00673-15

Zhang, H., Zhou, Z., Chen, J., Wu, G., Yang, Z., Zhou, Z., et al. (2014). Lack of long lasting hydrosalpinx in a/J mice correlates with rapid but transient chlamydial Ascension and neutrophil recruitment in the oviduct following intravaginal inoculation with *Chlamydia muridarum*. *Infect. Immun.* 82, 2688–2696. doi: 10.1128/IAI.00055-14

Zhong, G. (2017). Chlamydial plasmid-dependent pathogenicity. *Trends Microbiol.* 25, 141–152. doi: 10.1016/j.tim.2016.09.006

Zhong, G. (2018). Chlamydia spreading from the genital tract to the gastrointestinal tract - a two-hit hypothesis. *Trends Microbiol.* 26, 611–623. doi: 10.1016/j.tim.2017.12.002

Ziklo, N., Huston, W. M., Hocking, J. S., and Timms, P. (2016). Chlamydia trachomatis genital tract infections: when host immune response and the microbiome collide. *Trends Microbiol.* 24, 750–765. doi: 10.1016/j.tim.2016.05.007

Ziklo, N., Huston, W. M., Taing, K., Katouli, M., and Timms, P. (2016). In vitro rescue of genital strains of chlamydia trachomatis from interferon-gamma and tryptophan depletion with indole-positive, but not indole-negative Prevotella spp. *BMC Microbiol.* 16:286. doi: 10.1186/s12866-016-0903-4

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EDITED BY
Tingtao Chen,
Nanchang University, China

REVIEWED BY
Chuanyin Li,
Chinese Academy of Medical Sciences and Peking Union Medical College, China
Hao Cheng,
Zhejiang University, China
Qiongqiong Zhang,
Tsinghua University, China
Xia He,
Nanchang University, China

*CORRESPONDENCE
Min Hao
2yuanhaomin@163.com

†These authors have contributed equally to this work

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Interactions between vaginal local cytokine IL-2 and high-risk human papillomavirus infection with cervical intraepithelial neoplasia in a Chinese population-based study

Ruoxi Zhu[1†], Wenhao Wang[1†], Aiming Yang[2,3†], Weihong Zhao[1], Wei Wang[1], Zhilian Wang[1], Jintao Wang[4], Yongli Hou[1], Xiaoqiang Su[1], Lili Zhang[1], Bo Feng[1], Jing Yang[1], Zhe Wang[1], Xiaofen Niu[1], Weiguo Lv[5], Zhican Qu[1] and Min Hao[1*]

[1]Departments of Obstetrics and Gynecology, Second Hospital of Shanxi Medical University, Taiyuan, Shanxi, China, [2]Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, Hong Kong SAR, China, [3]Hong Kong Institute of Diabetes and Obesity, The Chinese University of Hong Kong, Hong Kong, Hong Kong SAR, China, [4]Department of Epidemiology, School of Public Health, Shanxi Medical University, Taiyuan, Shanxi, China, [5]Department of Gynecologic Oncology, Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China

Background: Although interleukin-2 (IL-2) has long been associated with cancer development, its roles in the development of cervical cancer remains unclear. Few studies examined the associations between IL-2 and high-risk human papillomavirus (HPV) with risk of cervical intraepithelial neoplasia (CIN).

Objective: We aimed to assess the association of IL-2 and high-risk HPV infection with risk of CIN as well as their interactions on the risk of CIN.

Design: We performed a cross-sectional analysis of screening data in 2285 women aged 19-65 years who participated in an ongoing community-based cohort of 40,000 women in Shanxi, China in 2014-2015. Both categorical and spline analyses were used to evaluation the association between IL-2 in the local vaginal fluids and prevalence of CIN. In addition, 1503 controls were followed up until January 31, 2019), the nested case-control study design was adopted to evaluate the association of vaginal lavage IL-2 levels and the risk of CIN progression.

Results: After adjusting for potential confounders, IL-2 levels were statistically inversely associated with prevalence of CIN (the 1st versus 4th quartile IL-2 levels: the respective odds ratio [OR] and 95% confidence intervals [CI] was: = 1.75 [1.37, 2.23] for CIN, 1.32 [1.01, 1.73] for CIN I, and 3.53 [2.26, 5.52] for CIN II/III). Increased IL-2 levels were inversely associated with prevalence of CIN (P-overall<0.01, P-nonlinearity<0.01 for CIN; P-overall<0.01, P-nonlinearity = 0.01 for CIN I; P-overall <0.01, P-nonlinearity = 0.62 for CIN II/III). The highest prevalence of CIN was observed in women with high-risk HPV, who also had the lowest IL-2 levels (P-interaction < 0.01). Nested case-control study observed

an inverse association between IL-2 levels and risk of CIN progression (OR=3.43, [1.17, 10.03]).

Conclusions: IL-2 levels in the local vaginal fluids were inversely associated with the risk of CIN in Chinese women either with or without high-risk HPV infection.

KEYWORDS

cervical intraepithelial neoplasia, cytokines, IL-2, high-risk human papillomavirus infection, cervical cancer

1 Introduction

Globally, the incidence of cervical cancer has declined due to the development of cervical cancer screening (Siegel et al., 2022). However, there has been an increasing trend in the incidence of cervical intraepithelial neoplasia (CIN) (Burness et al., 2020; Chen et al., 2020; Zhang et al., 2020). CIN is a precancerous lesion that pathologically reflects the dynamic changes of epithelial hyperplasia in cervical cancer and studying its mechanism is crucial for the prevention of cervical cancer (Curty et al., 2019; Kyrgiou and Moscicki, 2022; Kyrgiou et al., 2022). Previous studies have established the causal role of high-risk human papilloma virus (HPV) infection in the pathogenesis of CIN and cervical cancer (Bosch and de Sanjosé, 2003). However, in most women, the virus and diseased cells can be eliminated through autoimmune reactions, and only a few persistent infections eventually progress into cervical cancer. The immune system plays an important regulatory role in the development of CIN and cervical cancer caused by high-risk HPV infections (Sasagawa et al., 2012), as the occurrence and development of malignant diseases are closely related to the immune system.

Cytokines are low-molecular-weight soluble proteins that act as a medium of the immune system. They are induced by immunogens or other factors that are stimulated by immune or other cells, and have various functions such as regulating the development and function of the body's immune and non-immune systems. HPV infection in the body is mainly depend on the cellular immunity of CD4+ T helper cells (Th) and cytotoxic T cells (CTL) to eliminate the virus (Brito et al., 2021). After the activation, CD4+ T cells differentiate into Th1 and Th2 cells under the stimulation of local cytokines. These cells secrete interleukin (IL)-2, IL-12, and interferon-γ (IFN-γ) that are mainly involved in cellular immunity. These cytokines can infiltrate in the female reproductive tract and participate in the regulation of the vaginal microenvironmental immune network. Changes in the immune network are closely related to the outcome of cervical lesions (Xu, 2014). IL-2 is an important cellular immune factor with anti-tumor effects (Jiang et al., 2016; Mu et al., 2020). It has been approved for the treatment of metastatic renal cell carcinoma and melanoma (Daniilidis et al., 2016). Recent reports have also demonstrated that changes in IL-2 levels play an important role in the development and progression of cervical cancer (Pang et al., 2014; Valle-Mendiola et al., 2016; Zhao et al., 2016). However, the specific mechanism remains unclear. It is unclear whether IL-2 and high-risk HPV have synergistic effects in the development of CIN and cervical cancer. Therefore, exploring the associations of cytokine IL-2 levels and CIN risk, and synergy with the high-risk HPV infection is important in timely blocking CIN progression.

Vaginal microecology includes vaginal flora, endocrine regulation, local immunity, and anatomy. Local immunity may play an important role in maintaining microecological balance. As the cervix is located in the vagina, the vaginal micro-ecological imbalance can have a direct impact on the cervix. Local immunity may also have a more significant role in cervical lesions than systemic immunity. Moreover, as the cervix can be easily exposed; it can easily adjust local immunity and prevent disease progression. However, limited research has been conducted on the effects of local immunity on diseases; Therefore, measuring the cytokine IL-2 levels in the local vaginal fluids can be an effective way of assessing immune changes that accurately reflect the cervix's environment. This approach may prove to be more sensitive and specific than the blood and other body fluids.

To better understand the potential risk factors associated with CIN and to develop effective strategies for preventing its progression to cervical cancer, we launched a large population-based cervical cancer screening program and a prospective cohort study (Shanxi CIN Cohort) in Shanxi, China in 2014-2015 (Yang et al., 2018). In the study, we comprehensively assessed the associations of IL-2 levels in the local vaginal fluids with prevalence of CIN and interactions between IL-2 levels and high-risk HPV infection on CIN risk.

2 Material and methods

2.1 Study population

This study was conducted based on the data obtained from the Shanxi CIN Cohort Study in 2014-2015. The rationale, design, and methods of the cohort have been detailed elsewhere(Yang et al., 2018; Wang et al., 2021; Wang et al., 2022). Briefly, free cervical cancer screening was conducted for eligible women permanently

residing in two counties of Shanxi Province, China between 2014 and 2015. A total of 40,000 women aged 19–65 years were included. All participants completed a demographic characteristic-related questionnaire and a Pap test according to a liquid-based cytology (LBC). All participants with abnormal Pap test results were referred for colposcopy and histopathological examination. Amongst 2,769 women diagnosed with atypical squamous cells of undetermined significance (ASC-US), 2,691 women underwent colposcopy and histopathological examination, and 78 (10 cases of abnormal gland cells and 68 cases of rejection) were excluded. Among the 2,691 women with pathological results, 1,890 tested negative, 564 with CIN grade 1 (CIN I), 171 with CIN II, 47 with CIN III, and 19 with cervical cancer. In this study, 19 cases of cervical cancer were excluded. Among the 1,890 women with negative colposcopy and pathological results, 1,503 were included in the final analysis and 387 women were excluded because they had not fully completed the three parts of the medical examination, i.e., an in-person interview, physical examination, and clinical examination. The basic characteristics of the included and excluded women with negative pathological results are presented in Supplementary Table 1. Thus, a total of 2,285 women aged 49.2 ± 9.0 years (mean± standard deviation [SD]) was included in the present analysis.

Additionally, 1503 women of the control group had followed up until January 3, 2019. Twenty-five women (16 CIN I, 6 CIN II, 3 CIN III) with abnormal biopsy results were selected as the case group. The research carried out a follow-up on women with negative pathological examination results. During the follow-up period, patients who progressed to CIN I, CIN II or CIN III were the case group, and those who did not progress to the control group were matched at a ratio of 1:3. The matching condition included no disease progression occurred on the latest follow-up date of the case group, age ± 1 year old, and high-risk HPV infection. We finally matched to 25 cases and 75 controls. The study was approved by the ethical committees of the Second Hospital of Shanxi Medical University, and registered in the Chinese Clinical Trial Register (ChiCTR) (registration number: ChiCTR-ROC-15006479).

2.2 Data collection

2.2.1 Demographic characteristics and collection of factors related to cervical lesions

Face-to-face interviews were conducted by trained interviewers using a standardized and structured questionnaire. The interviewers were trained to administer the questionnaire in a standardized style. The survey mainly includes the demographic characteristics, such as age, educational years, smoking, alcohol drinking, and yearly income; characteristics of cervical lesion-related factors, such as parity, first sexual intercourse age, and family history of cancer.

2.2.2 Specimen collection

2.2.2.1 Collection of cervical cell samples

All participants were instructed to abstain from sexual intercourse and not perform vaginal lavage or medications for 48 h prior to the sampling. After exposing the cervix, the cervical detachment cells were collected by rotating the cervical cell brush five times, and the sampler was rinsed 10 times with the preservation solution, and then tightened after capping. The samples were stored at 4°C and tested within 1 week. Dissatisfactory cervical cell specimens were re-acquired after reading the films, and satisfactory specimens were obtained from each participant.

2.2.2.2 Operation procedure of HPV classification test specimen collection

The HPV test special brush was inserted into the cervical canal, and gently rotated for 3-5 turns; the brush head was left in the sample tube, and stored in −20°C refrigerator for inspection.

2.2.2.3 Vaginal lavage fluid collection

The vaginal lavage fluid was taken before the colposcopy biopsy. The speculum was opened, 5 ml of normal saline was taken using a disposable sterile syringe, 1/3 of the vagina and cervix was rinsed, and 5 ml of vaginal lavage was extracted, stored in a sterile centrifuge tube, and tested for the cytokine IL-2 levels. The collected vaginal lavage fluid was centrifuged for 10 min (2000 rpm), and the supernatant was dispensed and stored inside a refrigerator at −80°C until use.

2.2.2.4 Cervical tissue

Cervical tissue specimens were collected by a physician with colposcopy. The cervical mucus was gently wiped using a sterile cotton ball, and the suspicious part of cervix was taken using a colposcopy sterile biopsy forceps. The clamped tissue was immediately sent to the pathology department of our hospital for pathological diagnosis.

2.2.3 Clinical laboratory tests

2.2.3.1 Cervical Pap cytology specimen testing

All Pap tests were performed using an LBC method. Two cytopathologists from the Second Hospital of Shanxi Medical University performed the cytologic evaluation according to Bethesda System 2001 terminology. All slides with abnormal cytological results were further reviewed by a senior cytopathologist blinded on the previous pathological results for quality control.

2.2.3.2 Colposcopy and cervical histological examination

Colposcopy was performed by gynecological specialists at the Second Hospital of Shanxi Medical University according to a standard protocol, within 12 weeks from the Pap test. Video colposcopy was performed using the SLC-2000 device (Shenzhen Goldway Company, Shenzhen, Guangdong, China). During the colposcopy, the cervix was divided into quadrants, and each quadrant was examined. All visually abnormal areas were biopsied, and the quadrants without a visible lesion were biopsied at the squamocolumnar junction (“random biopsy”). Women with abnormal cytological results and negative or inadequate colposcopic findings also underwent endocervical curettage. Cervical biopsy and endocervical curettage results were evaluated by two gynecologic pathologists from the Second Hospital of Shanxi Medical

University. The cases were classified as negative, CIN I, CIN II, CIN III, or squamous cell carcinoma. The pathologists were blinded to the Pap test results when they read the cervical biopsy or endocervical curettage tissue specimens. If the two pathologists had different diagnoses, the cases were reviewed by a third senior pathologist. The three pathologists reviewed difficult or equivocal cases together to reach a consensus diagnosis.

2.2.3.3 HPV typing test

HPV genotyping by HybriMax was performed using an HPV GenoArray Test Kit (HybriBio Ltd) with the residual Pap test specimens. HPV types were divided into high-risk HPV types and others (including low-risk HPV types and negative) based on their oncogenic potential. This assay can identify 21 types of HPV, including 15 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68) and 6 low-risk (6, 11, 42, 43, 44, and CP8304) types through the use of a flow-through hybridization technique performed with a TC-96/G/H6 HPV DNA Amplification Analyzer and an HMM-2 fast nucleic acid molecule hybridization instrument (HybriBio Ltd).

2.2.3.4 IL-2 test

IL-2 levels were measured using the enzyme-linked immunosorbent assay (ELISA) kits (Jinma company in Shanghai) according to the manufacturer's instructions.

2.3 Statistical analysis

The EpiData 3.1 software was used to enter and manage the datasets. Descriptive statistics were used to describe the frequency, proportion, mean, median, and quartile and the characteristics of demographic and cervical lesion-related factors. Participants' characteristics were examined for significant difference(s) using the Pearson's chi-square test for categorical variables and t-test for continuous variables. Logistic regression models were used to test odds ratios (ORs) and confidence intervals (CIs) of CIN II/III for each IL-2 quartile compared with the highest quartile. Tests of linear trends across the increasing quartiles of IL-2 levels were conducted by assigning the medians of quartiles treated as continuous variables. Interaction between IL-2 levels and high-risk HPV infections with prevalence of CIN were evaluated by including the terms in the model. After the crude analysis, we adjusted for the demographic characteristics such as age, educational level, yearly income, smoking, alcohol drinking, parity, first sexual intercourse age, family history of cancer, and high-risk HPV infection fitted simultaneously. Smoking was defined as those who smoked at least 1 cigarette/day in the past 6 months. Alcohol drinking was defined as those who drank hard liquor, beer, or wine at least 1/week in the past 6 months.

We further performed the spline analysis with a three-knot (25th, 50th, and 75th percentiles) restricted cubic spline function (Desquilbet and Mariotti, 2010; Yang et al., 2018) to determine the dose-response association between log-transformed IL-2 levels and prevalence of CIN. All statistical analyses were performed with SAS software version 9.3 (SAS Institute Inc.). All reported P-values were derived based on the two-sided tests with a significance level of 0.05.

3 Results

Table 1 shows the characteristics of 2,285 women who underwent a cervical histologic examination. The proportion of women with CIN accounted for 34.2% of the analyzed participants including 564 with CIN I, 171 with CIN II, and 47 with CIN III, The respective proportion in women with and without CIN were 64.3% and 68.7% age above 45 years, 23.1% and 19.2% for yearly income above 30,000 yuan, 2.4% and 4.4% for alcohol drinking, and 13.3% and 8.8% for family history of cancer. Women with CIN were more likely infected with high-risk HPV versus those without CIN (39.5% versus 28.5%). Amongst 782 women with CIN, 72.1% were CIN I with high-risk HPV (n=564) with high-risk HPV prevalence of 29.6%, only 27.9% of women were in CIN II+ (n=218) with the prevalence of high-risk HPV of 65.1%. The median IL-2 level in the vaginal lavage fluid among women with and without CIN was 171.00 (132.93–221.22) pg/ml and 186.00 (151.00–226.38) pg/ml, respectively.

Table 2 presents the associations of IL-2 levels and high-risk HPV infection with prevalence of CIN in 2,285 women. After fully adjusting the demographic, lifestyle, and other covariates including high-risk HPV infection, IL-2 levels were inversely associated with prevalence of CIN. The 1st quartile levels of IL-2 had a 75% increased odds of CIN than the 4th quartile (OR=1.75, 95% CI: 1.37, 2.23, P-trend < 0.01). Compared with women without high-risk HPV infections, those with high-risk HPV infection had an increased odds of CIN (OR = 1.58, 95% CI: 1.31, 1.90). In stratified analysis by CIN subgroups, a statistically significant association between IL-2 levels and prevalence of CIN I was observed (1st versus 4th quartile: OR = 1.32; 95% CI: 1.01 1.73) while an inverse association was observed for CIN II/III (1st versus 4th quartile: OR = 3.53; 95% CI: 2.26, 5.52).

Among the 2285 women, 1563 were high-risk HPV negative, and 15 of them were low-risk HPV infected. The mean ± SD of IL-2 in 15 women with low-risk HPV infection was 181.64 ± 49.46pg/ml with the median was 178.55pg/ml. Among 722 women with high-risk HPV infections the mean ± SD of 194.58 ± 99.86pg/ml; 1548 negative cases, with a mean ± SD of 204.82 ± 108.21pg/ml and a median of 183.60 pg/ml. We did not observe he statistically significant difference between negative, low-risk HPV and high-risk HPV patients (P = 0.075). After adjusting for all potential confounders including high-risk HPV infections, we observed the inverse linear dose-response associations between IL-2 levels and prevalence of CIN (P overall <0.01, P nonlinear < 0.01, CIN I), CIN I (P overall <0.01, P nonlinear= 0.01), and CIN II/III (P overall <0.01, P nonlinear = 0.62) (Figure 1).

The highest prevalence of CIN was observed in women with high-risk HPV infections, who also had the lowest IL-2 levels (≤144.26 pg/ml; OR = 2.88, 95% CI: 2.02, 4.11) (Figure 2). IL-2 was associated with a lower prevalence of high-risk HPV infection in Chinese women of this cohort (OR = 0.99; 95% CI: 0.99, 1.00; P=0.03) (Supplementary Table 2).

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TABLE 1 Characteristics of 2,285 women with cervical histologic examination[1] (%).

Characteristics	CIN		Total	*P value*[2]
	No	Yes		
No. of participants	1503 (65.8)	782 (34.2)	2285 (100.0)	
Age, years	49.4 ± 8.9	48.6 ± 9.2	49.2 ± 9.0	
≤ 45	470 (31.3)	279 (35.7)	211 (9.2)	0.03
>45	1033 (68.7)	503 (64.3)	636 (27.9)	
Education, years				
≤9	1259 (86.2)	675 (86.3)	1970 (86.2)	0.16
>9	208 (13.8)	107 (13.7)	315 (13.8)	
Yearly income, ¥				
<30000	1214 (80.8)	601 (76.9)	1815 (79.4)	0.03
≥30000	289 (19.2)	181 (23.1)	470 (20.6)	
Smoking				
No	1474 (98.1)	763 (97.6)	2237 (97.9)	0.43
Yes	29 (1.9)	19 (2.4)	48 (2.1)	
Alcohol drinking				
No	1437 (95.6)	763 (97.6)	2200 (96.3)	0.02
Yes	66 (4.4)	19 (2.4)	85 (3.7)	
Parity				
<3	1110 (79.3)	578 (73.9)	1688 (73.9)	0.98
≥3	393 (26.1)	204 (26.1)	597 (26.1)	
First sexual intercourse age, years				
<23	847 (56.4)	464 (59.3)	1311 (57.4)	0.17
≥23	656 (43.6)	318 (40.7)	974 (42.6)	
Family history of cancer				
No	1370 (91.2)	678 (86.7)	2048 (89.6)	0.01
Yes	133 (8.8)	104 (13.3)	237 (10.4)	
High-risk HPV infection				
Negative	1075 (71.5)	473 (60.5)	1548 (67.7)	<0.01
Positive	428 (28.5)	309 (39.5)	737 (32.3)	
IL-2 (pg/ml)	186.00 (151.00-226.38)	171.00 (132.93-221.22)	182.00 (144.26-224.90)	<0.01

[1]Values are mean ± SD for normally distributed variables, median (25–75 percentiles) for skewed variables, or n (%) for categoric variables. CIN, cervical intraepithelial neoplasia
[2]P values for differences between groups were obtained from the chi-square test for categoric variables, and t-test for continuous variables.

In the nested case-control study, we compared IL-2 expression levels before and after follow-up. The mean ± SD of IL-2 level before follow-up in the progression group (n=25) was 205.4 ± 93.84 pg/ml, the median value was 202.0 pg/ml. The mean ± SD IL-2 level at end of follow-up was 142.0 ± 58.44 pg/ml (media= 137.0 pg/ml). The mean ± SD of IL-2 level before follow-up in the non-progressive population (n=75) was 207.1 ± 123.8 pg/ml (median = 177.0 pg/ml). The mean ± SD of IL-2 level at end of follow-up was 170.3 ± 42.11 pg/ml (median=172.0 pg/ml). After adjusting the potential confounders, those with local vaginal IL-2 ≤ 169.00pg/ml (median) had a 3.43 (1.17-10.03) times risk of progression to CIN than those with IL-2>169.00pg/ml (Table 3). We also observed that IL-2 was inversely associated with risk of high-risk HPV infection (OR = 0.20; 95% CI: 0.09, 0.47) (Supplementary Table 3).

4 Discussion

In this large-scale population-based study, we found that IL-2 levels in the local vaginal were associated with higher prevalence of

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TABLE 2 The associations between IL-2 levels and high-risk HPV infection with prevalence of cervical intraepithelial neoplasia (CIN)among 2,285 women[1].

	Odds Ratios (95% CIs)[2]		
	Model 1	Model 2	Model 3
CIN			
IL-2 (pg/ml)			
Q1 (≤ 144.26)	1.72 (1.35-2.19)	1.78 (1.39-2.26)	1.75 (1.37-2.23)
Q2 (144.27-182.00)	0.96 (0.75-1.24)	1.02 (0.79-1.31)	1.00 (0.77-1.28)
Q3 (182.01-224.90)	0.77 (0.59-0.99)	0.79 (0.61-1.02)	0.79 (0.61-1.02)
Q4 (≥ 224.91)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
P- trend	< 0.01	< 0.01	< 0.01
High-risk HPV infection			
Positive	1.64 (1.37-1.97)	1.61 (1.33-1.93)	1.58 (1.31-1.90)
Negative	1.00 (reference)	1.00 (reference)	1.00 (reference)
CIN I			
IL-2 (pg/ml)			
Q1 (≤ 144.26)	1.32 (1.01-1.72)	1.33 (1.02-1.73)	1.32 (1.01-1.73)
Q2 (144.27-182.00)	0.85 (0.65-1.12)	0.86 (0.66-1.14)	0.86 (0.66-1.14)
Q3 (182.01-224.90)	0.67 (0.51-0.89)	0.68 (0.51-0.90)	0.68 (0.51-0.90)
Q4 (≥ 224.91)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
P- trend	0.08	0.07	0.07
High-risk HPV infection			
Positive	1.06 (0.86-1.31)	1.05 (0.85-1.31)	1.04 (0.83-1.29)
Negitive	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
CIN II/III			
IL-2 (pg/ml)			
Q1 (≤ 144.26)	3.64 (2.37-5.58)	3.69 (2.40-5.70)	3.53 (2.26-5.52)
Q2 (144.27-182.00)	1.51 (0.95-2.41)	1.59 (0.99-2.56)	1.48 (0.91-2.41)
Q3 (182.01-224.90)	1.22 (0.76-1.98)	1.28 (0.78-2.07)	1.27 (0.77-2.09)
Q4 (≥ 224.91)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
P- trend	< 0.01	< 0.01	< 0.01
High-risk HPV infection			
Positive	4.69 (3.48-6.31)	4.31 (3.18-5.86)	4.39 (3.20-6.01)
Negative	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)

[1]Values are ORs (95% CIs) obtained from logistic regression analysis, using the highest intake group as the reference, unless otherwise indicated. CIN, cervical intraepithelial neoplasia.
[2]Model 1: unadjusted.
Model 2: adjusted for age, educational level, yearly income, smoking, alcohol drinking, parity, first sexual intercourse age, and family history of cancer
Model 3: adjusted for Model 2 + high-risk HPV infection

CIN, with a non-linear dose-response associations in Chinese women in the Shanxi CIN cohort. Women with high-risk HPV infections and the lowest IL-2 levels in the vaginal lavage fluid had the highest prevalence of CIN. These results indicate the potential role of IL-2 levels in vaginal lavage in the development of CIN, regardless of high-risk HPV infections.

Previous studies on CIN have mostly focused on systemic immunity, however, studies on the cervical microenvironment have been limited. The mucosal immunity of the genitals is the first line of defense in the female genital tract. It has cellular and humoral immunity that is regulated by pathogens and cytokines. As an important component of the body's immunity, the expression of

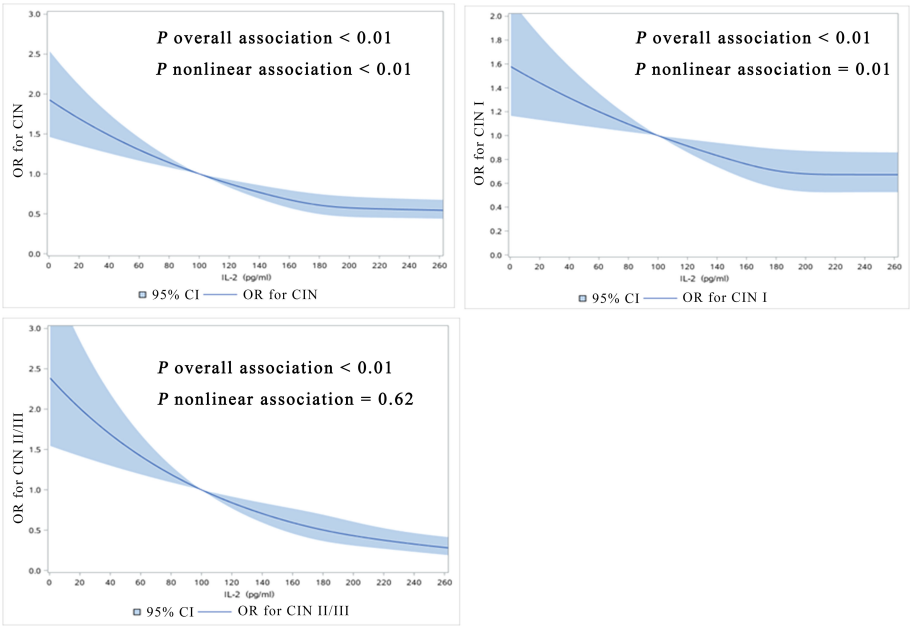

FIGURE 1
Dose-response relationship between IL-2 levels with prevalence of CIN, CIN I, CIN II/III (restricted cubic spline models). The solid line represents the OR from the adjusted restricted cubic polynomial spline. The shaded area is the 95% CI. Adjusted for age, educational level, yearly income, smoking, alcohol drinking, parity, first sexual intercourse age, family history of cancer, and high-risk HPV infection. CIN, cervical intraepithelial neoplasia.

local cytokines can better reflect the local immune status of the genital tract (Mitra et al., 2021). The incidence of CIN and cervical cancer is associated with high-risk HPV infection, and local immune capacity and systemic immunity may change after high-risk HPV infection (Arany et al., 2002; Torres-Poveda et al., 2016). cytokines have a high affinity for cell surface receptors, serving as an important structural basis for their biological function. *Passmore J A et al.* (Passmore et al., 2006) showed that local cytokines are more representative of local immune status than serum cytokines. Furthermore, vaginal lavage fluid collection is noninvasive, and specimens are readily available.

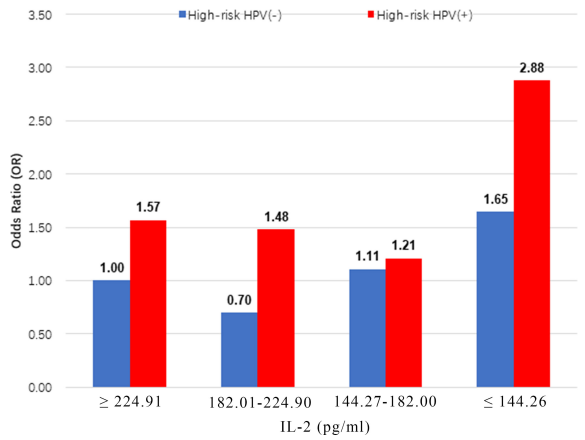

FIGURE 2
Multiplicative interaction between IL-2 level and high-risk HPV infection with prevalence of CIN. ORs are adjusted by age, educational level, yearly income, smoking, alcohol drinking, parity, first sexual intercourse age, family history of cancer.IL-2 ORs for CIN: 1.00 (reference), 0.70 (95% CI: 0.51, 0.96), 1.11 (95% CI: 0.81, 1.51), 1.65 (95% CI: 1.22, 2.22), 1.57 (95% CI: 1.08, 2.29), 1.48 (95% CI: 1.01, 2.17), 1.21 (95% CI: 0.83, 1.76) and 2.88 (95% CI: 2.02,4.11). CIN, cervical intraepithelial neoplasia.

4.1 Association between IL-2 level and CIN risk

The role of IL-2 in the development of CIN and cervical cancer remains unclear (Meng and Song, 2019; Chen et al., 2021). Evidence from previous studies on the association of increased IL-2 level with decreased risk of CIN were certainly consistent. IL-2 can induce the differentiation of natural killer cells (NK) and CTL and exert anti-tumor effects (Banchereau et al., 2012). The activity of NK and CTL is decreased in the absence of IL-2, promoting the development of tumors including cervical cancer (Lieberman and Tsokos, 2010; Valle-Mendiola et al., 2016; Abel et al., 2018). Studies have shown that IL-2 inhibits the proliferation of cervical cancer cells at high doses (Valle-Mendiola et al., 2014). A recent study showed that serum IL-2 levels were significantly reduced in high-grade CIN compared with low-grade CIN (Daniilidis et al., 2016). Our study using baseline data also showed that even after adjusting for high-risk HPV and other potential confounders, vaginal local IL-2 was associated with the odds of CIN (OR=1.75 [1.37-2.23]). In addition, women with lower vaginal IL-2 levels were also more likely to develop into CIN in our follow-up data.

IL-2 has been widely used as a treatment for several other tumors by promoting CTL proliferation and activity (Kang et al., 2012; Abel et al., 2018; Dutcher and Wiernik, 2018; Ngai et al., 2018;

TABLE 3 Logistic regression analysis of IL-2 and risk of CIN in follow-up (n=100).

	ORs (95% CIs)[1]
IL-2 (pg/ml)	
≤ 169.00 (median)	3.43 (1.17-10.03)
>169.00 (median)	1.00 (Reference)

[1]adjusted for age, educational level, yearly income, smoking, alcohol drinking, parity, first sexual intercourse age, and family history of cancer.

Rolley et al., 2018). Thus, the specific role of IL-2 is also being explored in cervical cancer. However, whether low-level IL-2 promotes the occurrence of CIN remains unclear. Furthermore, the definition of the low level of IL-2 is not well-defined. More research is needed to confirm these results.

4.2 Interactions between cytokine IL-2 levels and high-risk HPV infection

High-risk HPV infection is an essential factor in the development of cervical cancer (Yu et al., 2022). In this study, we observed a lower levels of IL-2 in vaginal lavage fluids and the interaction of high-risk HPV infection with the risk of CIN. The association between IL-2 levels and the development of CIN and the interaction between IL-2 levels and high-risk HPV infection has not been fully elucidated in epidemiological studies. The exact associations between IL-2 levels and high-risk HPV infection in the vaginal microenvironment with the development of cervical cancer remains unclear. Most HPV infections are transient, and 90% of HPV-DNA is negative in women with normal immune function after 2 years. Even in women with CIN, high-risk HPV infection has a higher natural outcome rate if followed up for a sufficient period of time. After the high-risk HPV infection, the virus is mainly eliminated by the body's immune response, and IL-2 is the most prominent anti-infective (Barros et al., 2018). IL-2 has been shown to act as an adjuvant vaccine to enhance mucosal immunity and form long-term memory responses (Kim et al., 2013; Ma et al., 2015). Studies by *Bashaw et al.* (Bashaw et al., 2017) showed that IL-2 may play an anti-high-risk HPV role in the early stage of infection. When IL-2 level decreases, it may lead to the decline of host anti-tumor immunity, which is not conducive to clearing HPV infection, and then leads to disease progression. *Paradkar et al.* (Paradkar et al., 2014) reported that IL-2 levels were lower in the peripheral blood of women with high-risk HPV infection than those without.

The studies mentioned above, decreased IL-2 levels in the vaginal microenvironment may affect the clearance of high-risk HPV and lead to persistent infection of high-risk HPV, while persistent infection of high-risk HPV may cause the decreased secretion of IL-2 by local production of E6, E7, etc. (Trujillo-Cirilo et al., 2021), which promotes the development of cervical cancer. Such interactions may be one of the underlying mechanisms that promote the development of CIN and cervical cancer.

Several advantages and limitations should be considered when interpreting these results. First, this large-scale study ensures that statistically significant differences can be detected. Second, an objective assessment of IL-2 levels, high-risk HPV, and other cervical cancer-related clinical examinations such as Pap test colposcopy, and cervical biopsy was conducted. Pathological results regarded as the "golden standard" instead of general cytology were used in classifying the participants. Third, potential confounding factors were carefully and comprehensively measured and analyzed to minimize the possibility of bias. Fourth, the lower high-risk HPV positive rate for CIN patients in this study may be due to differences in the study population or variations in pathological diagnosis and laboratory examination. Lastly, the dose-response relationship between IL-2 levels and CIN as well as their interactions based on high-risk HPV infection were evaluated. The main limitation of this study is that the participants were regionally confined. Since the information in this study is based on the screening and investigation of group research in Shanxi Province, regional differences could not be overcome.

In summary, IL-2 levels in local vaginal fluid were independently associated with risk of CIN in this population-based study. Given the significant interactions between IL-2 and high-risk HPV infection with CIN risk, future large-scale prospective studies should focus on developing a strategy to determine these interactions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was approved by the ethical committees of the Second Hospital of Shanxi Medical University, and registered in the Chinese Clinical Trial Register (ChiCTR) (registration number: ChiCTR-ROC-15006479). The patients/participants provided their written informed consent to participate in this study.

Author contributions

RZ and WHW designed the analysis pipeline and wrote the manuscript. AY revised the manuscript critically. WZ, WW, ZLW, and JW performed atherosclerotic plaque collection and sorting. BF, JY, ZW, and XN created figures. MH reviewed and revised the paper in detail. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer HC declared a shared affiliation, with no collaboration, with one of the authors, WL, to the handling editor at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1109741/full#supplementary-material

References

Abel, A. M., Yang, C., Thakar, M. S., and Malarkannan, S. (2018). Natural killer cells: development, maturation, and clinical utilization. *Front. Immunol.* 9. doi: 10.3389/fimmu.2018.01869

Arany, I., Grattendick, K. G., and Tyring, S. K. (2002). Interleukin-10 induces transcription of the early promoter of human papillomavirus type 16 (HPV16) through the 5'-segment of the upstream regulatory region (URR). *Antiviral Res.* 55 (2), 331–339. doi: 10.1016/s0166-3542(02)00070-0

Banchereau, J., Pascual, V., and O'Garra, A. (2012). From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. *Nat. Immunol.* 13 (10), 925–931. doi: 10.1038/ni.2406

Barros, M. R.Jr., de Oliveira, T. H. A., de Melo, C. M. L., Venuti, A., and de Freitas, A. C. (2018). Viral modulation of TLRs and cytokines and the related immunotherapies for HPV-associated cancers. *J. Immunol. Res.* 2018, 2912671. doi: 10.1155/2018/2912671

Bashaw, A. A., Leggatt, G. R., Chandra, J., Tuong, Z. K., and Frazer, I. H. (2017). Modulation of antigen presenting cell functions during chronic HPV infection. *Papillomavirus Res.* 4, 58–65. doi: 10.1016/j.pvr.2017.08.002

Bosch, F. X., and de Sanjosé, S. (2003). Chapter 1: human papillomavirus and cervical cancer–burden and assessment of causality. *J. Natl. Cancer Inst Monogr.* 31), 3–13. doi: 10.1093/oxfordjournals.jncimonographs.a003479

Brito, M. J., Sequeira, P., Silva, I., Quintas, A., Martins, C., and Félix, A. (2021). CD4(+) and CD8(+) cell populations in HIV-positive women with cervical squamous intraepithelial lesions and squamous cell carcinoma. *Int. J. Infect. Dis.* 103, 370–377. doi: 10.1016/j.ijid.2020.10.083

Burness, J. V., Schroeder, J. M., and Warren, J. B. (2020). Cervical colposcopy: indications and risk assessment. *Am. Fam Physician* 102 (1), 39–48.

Chen, Z., Dong, D., Zhu, Y., Pang, N., and Ding, J. (2021). The role of Tim-3/Galectin-9 pathway in T-cell function and prognosis of patients with human papilloma virus-associated cervical carcinoma. *FASEB J.* 35 (3), e21401. doi: 10.1096/fj.202000528RR

Chen, Y., Qiu, X., Wang, W., Li, D., Wu, A., Hong, Z., et al. (2020). Human papillomavirus infection and cervical intraepithelial neoplasia progression are associated with increased vaginal microbiome diversity in a Chinese cohort. *BMC Infect. Dis.* 20 (1), 629. doi: 10.1186/s12879-020-05324-9

Curty, G., de Carvalho, P. S., and Soares, M. A. (2019). The role of the cervicovaginal microbiome on the genesis and as a biomarker of premalignant cervical intraepithelial neoplasia and invasive cervical cancer. *Int. J. Mol. Sci.* 21 (1), 222. doi: 10.3390/ijms21010222

Daniilidis, A., Koutsos, J., Oikonomou, Z., Nasioutziki, M., Hatziparadisi, K., and Tantanasis, T. (2016). Cytokines of cervical mucosa and human papilloma virus infection of the cervix: a descriptive study. *Acta Cytol* 60 (1), 58–64. doi: 10.1159/000445161

Desquilbet, L., and Mariotti, F. (2010). Dose-response analyses using restricted cubic spline functions in public health research. *Stat. Med.* 29 (9), 1037–1057. doi: 10.1002/sim.3841

Dutcher, J. P., and Wiernik, P. H. (2018). Interleukin-2 can cure kidney cancer. *Oncologist* 23 (9), e107. doi: 10.1634/theoncologist.2018-0038

Jiang, T., Zhou, C., and Ren, S. (2016). Role of IL-2 in cancer immunotherapy. *Oncoimmunology* 5 (6), e1163462. doi: 10.1080/2162402x.2016.1163462

Kang, T. H., Mao, C. P., He, L., Tsai, Y. C., Liu, K., La, V., et al. (2012). Tumor-targeted delivery of IL-2 by NKG2D leads to accumulation of antigen-specific CD8+ T cells in the tumor loci and enhanced anti-tumor effects. *PloS One* 7 (4), e35141. doi: 10.1371/journal.pone.0035141

Kim, H., Kwon, B., and Sin, J. I. (2013). Combined stimulation of IL-2 and 4-1BB receptors augments the antitumor activity of E7 DNA vaccines by increasing Ag-specific CTL responses. *PloS One* 8 (12), e83765. doi: 10.1371/journal.pone.0083765

Kyrgiou, M., Athanasiou, A., Arbyn, M., Lax, S. F., Raspollini, M. R., Nieminen, P., et al. (2022). Terminology for cone dimensions after local conservative treatment for cervical intraepithelial neoplasia and early invasive cervical cancer: 2022 consensus recommendations from ESGO, EFC, IFCPC, and ESP. *Lancet Oncol.* 23 (8), e385–e392. doi: 10.1016/s1470-2045(22)00191-7

Kyrgiou, M., and Moscicki, A. B. (2022). Vaginal microbiome and cervical cancer *Semin Cancer Biol* 86 (3), 189–198. doi: 10.1016/j.semcancer.2022.03.005

Lieberman, L. A., and Tsokos, G. C. (2010). The IL-2 defect in systemic lupus erythematosus disease has an expansive effect on host immunity. *J. BioMed. Biotechnol.* 2010, 740619. doi: 10.1155/2010/740619

Ma, F., Zhang, Q., and Zheng, L. (2015). Interleukin/chitosan (JY) adjuvant enhances the mucosal immunity of human papillomavirus 16 L1 virus-like particles in mice. *Biotechnol. Lett.* 37 (4), 773–777. doi: 10.1007/s10529-014-1739-3

Meng, J. W., and Song, J. H. (2019). Association between interleukin-2, interleukin-10, secretory immunoglobulin a and immunoglobulin G expression in vaginal fluid and human papilloma virus outcome in patients with cervical lesions. *Oncol. Lett.* 18 (5), 5543–5548. doi: 10.3892/ol.2019.10897

Mitra, A., MacIntyre, D. A., Paraskevaidi, M., Moscicki, A. B., Mahajan, V., Smith, A., et al. (2021). The vaginal microbiota and innate immunity after local excisional treatment for cervical intraepithelial neoplasia. *Genome Med.* 13 (1), 176. doi: 10.1186/s13073-021-00977-w

Mu, L., Miao, J. R., and Song, J. H. (2020). The correlation of HPV16 and HPV18 with local vaginal immunity after the treatment of cervical intraepithelial neoplasia. *Transl. Cancer Res.* 9 (7), 4212–4223. doi: 10.21037/tcr-19-2955

Ngai, H., Tian, G., Courtney, A. N., Ravari, S. B., Guo, L., Liu, B., et al. (2018). IL-21 selectively protects CD62L(+) NKT cells and enhances their effector functions for adoptive immunotherapy. *J. Immunol.* 201 (7), 2141–2153. doi: 10.4049/jimmunol.1800429

Pang, X., Zhang, Y., Wei, H., Zhang, J., Luo, Q., Huang, C., et al. (2014). Expression and effects of high-mobility group box 1 in cervical cancer. *Int. J. Mol. Sci.* 15 (5), 8699–8712. doi: 10.3390/ijms15058699

Paradkar, P. H., Joshi, J. V., Mertia, P. N., Agashe, S. V., and Vaidya, R. A. (2014). Role of cytokines in genesis, progression and prognosis of cervical cancer. *Asian Pac J. Cancer Prev.* 15 (9), 3851–3864. doi: 10.7314/apjcp.2014.15.9.3851

Passmore, J. A., Milner, M., Denny, L., Sampson, C., Marais, D. J., Allan, B., et al. (2006). Comparison of cervical and blood T-cell responses to human papillomavirus-16 in women with human papillomavirus-associated cervical intraepithelial neoplasia. *Immunology* 119 (4), 507–514. doi: 10.1111/j.1365-2567.2006.02465.x

Rolley, C., Aubert, C., Baize, N., and Bigot, P. (2018). Management of metastatic renal cell carcinomass. *Prog. Urol* 28 (14), 777–782. doi: 10.1016/j.purol.2018.07.280

Sasagawa, T., Takagi, H., and Makinoda, S. (2012). Immune responses against human papillomavirus (HPV) infection and evasion of host defense in cervical cancer. *J. Infect. Chemother.* 18 (6), 807–815. doi: 10.1007/s10156-012-0485-5

Siegel, R. L., Miller, K. D., Fuchs, H. E., and Jemal, A. (2022). Cancer statistic. *CA Cancer J. Clin.* 72 (1), 7–33. doi: 10.3322/caac.21708

Torres-Poveda, K., Burguete-García, A. I., Bahena-Román, M., Méndez-Martínez, R., Zurita-Díaz, M. A., López-Estrada, G., et al. (2016). Risk allelic load in Th2 and Th3 cytokines genes as biomarker of susceptibility to HPV-16 positive cervical cancer: a case control study. *BMC Cancer* 16, 330. doi: 10.1186/s12885-016-2364-4

Trujillo-Cirilo, L., Torres-Corioriles, E. I., Rangel-Corona, R., Corona-Ortega, M. T., and Weiss-Steider, B. (2021). Evidence that the viral oncoproteins E6 and E7 of HPV induce the expression of a functional IL-2R on cervical cancer cells. *Cytokine* 148, 155592. doi: 10.1016/j.cyto.2021.155592

Valle-Mendiola, A., Gutiérrez-Hoya, A., Lagunas-Cruz Mdel, C., Weiss-Steider, B., and Soto-Cruz, I. (2016). Pleiotropic effects of IL-2 on cancer: its role in cervical cancer. *Mediators Inflammation* 2016, 2849523. doi: 10.1155/2016/2849523

Valle-Mendiola, A., Weiss-Steider, B., Rocha-Zavaleta, L., and Soto-Cruz, I. (2014). IL-2 enhances cervical cancer cells proliferation and JAK3/STAT5 phosphorylation at low doses, while at high doses IL-2 has opposite effects. *Cancer Invest.* 32 (4), 115–125. doi: 10.3109/07357907.2014.883526

Wang, Z., Wang, W., Yang, A., Zhao, W., Yang, J., Wang, Z., et al. (2021). Lower dietary mineral intake is significantly associated with cervical cancer risk in a population-based cross-sectional study. *J. Cancer* 12 (1), 111–123. doi: 10.7150/jca.39806

Wang, W., Yang, A., Zhang, H., Li, Y., Yang, J., Zhao, W., et al. (2022). Associations of RBC and serum folate concentrations with cervical intraepithelial neoplasia and high-risk human papillomavirus genotypes in female Chinese adults. *J. Nutr.* 152 (2), 466–474. doi: 10.1093/jn/nxab396

Xu, H. M. (2014). Th1 cytokine-based immunotherapy for cancer. *Hepatobiliary Pancreat Dis. Int.* 13 (5), 482–494. doi: 10.1016/s1499-3872(14)60305-2

Yang, J., Yang, A., Wang, Z., Wang, W., Wang, Z., Wang, Y., et al. (2018). Interactions between serum folate and human papillomavirus with cervical intraepithelial neoplasia risk in a Chinese population-based study. *Am. J. Clin. Nutr.* 108 (5), 1034–1042. doi: 10.1093/ajcn/nqy160

Yu, L., Chen, X., Liu, X., Fei, L., Ma, H., Tian, T., et al. (2022). Significance of triple detection of p16/ki-67 dual-staining, liquid-based cytology and HR HPV testing in screening of cervical cancer: a retrospective study. *Front. Oncol.* 12. doi: 10.3389/fonc.2022.915418

Zhang, S., Saito, M., Yamada, S., Sakamoto, J., Takakura, M., Takagi, H., et al. (2020). The prevalence of VAIN, CIN, and related HPV genotypes in Japanese women with abnormal cytology. *J. Med. Virol.* 92 (3), 364–371. doi: 10.1002/jmv.25611

Zhao, W., Hao, M., Wang, Y., Feng, N., Wang, Z., Wang, W., et al. (2016). Association between folate status and cervical intraepithelial neoplasia. *Eur. J. Clin. Nutr.* 70 (7), 837–842. doi: 10.1038/ejcn.2016.35

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EDITED BY
Nahed Ismail,
University of Illinois Chicago, United States

REVIEWED BY
Zhao-Jia Ge,
Qingdao Agricultural University, China
Xinliang Chen,
International Peace Maternity and Child Health Hospital, China

*CORRESPONDENCE
Lei Zhang
18601130459@163.com
Qinping Liao
13701124527@163.com
Xiaowen Tong
Xiaowen_tong@hotmail.com

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Analysis of the microbiota composition in the genital tract of infertile patients with chronic endometritis or endometrial polyps

Junhua Liang[1], Meng Li[2], Lei Zhang[2*], Yang Yang[1], Xia Jin[1], Qiongqiong Zhang[2], Tao Lv[2], Zhenyu Huang[2], Qinping Liao[2*] and Xiaowen Tong[1*]

[1]Department of Obstetrics and Gynecology, Tongji Hospital, School of Medicine, Tongji University, Shanghai, China, [2]Department of Obstetrics and Gynecology, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing, China

Background: The previous researches show that infertile patients have a higher incidence of endometritis and endometrial polyps, and the occurrence of these two diseases is related to changes in the microbiota of the genital tract. We aim to determine the composition and changing characteristics of the microbiota in the genital tract (especially the endometrium) of infertile patients with chronic endometritis or endometrial polyps, and find the correlation between it and the occurrence of diseases.

Methods: This is a prospective study. We collected genital tract biopsy samples from 134 asymptomatic infertile patients receiving assisted reproductive therapy before embryo transfer. Through pathological examination and 16S ribosomal RNA(16S rRNA) sequencing, we determined the distribution of chronic endometritis and endometrial polyps in these patients, as well as their distribution of reproductive tract microorganisms.

Results: Compared with the normal control group, the microbial group of reproductive tract in patients with chronic endometritis and endometrial polyps is changed, and there are significant species differences and relative abundance differences in the vagina, cervix and uterine cavity. *Lactobacillus*, the dominant flora of female genital tract, showed a change in abundance in patients with endometrial diseases. Endometrial microbiota composed of *Staphylococcus, Gardnerella, Atopobium, Streptococcus, Peptostreptococcus, Chlamydia, Fusobacterium, Acinetobacter*, etc. are related to chronic endometritis and endometrial polyps.

Conclusion: The results showed that, compared with the normal control group, the endometrial microbiota of infertile patients with chronic endometritis or endometrial polyps did have significant changes in the relative abundance distribution of species, suggesting that changes in local microecology may be

an important factor in the occurrence of disease, or even adverse pregnancy outcomes. The further study of endometrial microecology may provide a new opportunity to further improve the diagnosis and treatment strategy of chronic endometritis.

KEYWORDS

microbiota composition, genital tract, infertile patients, chronic endometritis, endometrial polyps

Introduction

Chronic endometritis (CE) is a disease in which the endometrium is infected and the inflammatory reaction persists. Because of its atypical clinical symptoms, inconsistent diagnostic methods, and inappropriately targeted treatment, it is still a gynecological disease that is underestimated and ignored clinically. A survey on the understanding and management of CE by obstetricians and gynecologists in the United States shows that doctors have a series of worrying defects in the pathophysiology, clinical manifestations and diagnostic methods of CE, especially in the treatment of CE ([[NoAuthor]]). Because CE has no typical clinical manifestations and is difficult to diagnose, the actual prevalence of CE in the general population is not clear and fluctuates widely, estimated to be between 0.8% and 25.2%. Although there is no typical symptom free clinic, studies have found that CE will reduce the success rate of natural and assisted reproductive technology (ART) successful pregnancy, and lead to obstetric and neonatal complications (Miles et al., 2017). In addition, among women diagnosed with unexplained repeated abortion or repeated implantation failure, the CE incidence rate was as high as 60% or 66%, respectively (Kimura et al., 2019). Therefore, it is particularly important to deeply explore the characteristics of CE pathogenic microorganisms and explore effective diagnosis and treatment methods. In the past, it was generally believed that endometrial polyp (EP) was an inflammatory disease, or a manifestation of chronic endometritis, which was formed by the reactive proliferation of endometrium under long-term sustained mechanical stimulation and biological inflammatory factors. However, recent studies have shown that the occurrence of EPs may involve many factors, such as the expression imbalance of estrogen receptor (ER) and progesterone receptor (PR), long-term sustained high level estrogen stimulation, abnormal cell apoptosis and proliferation, gene mutation, local endometrial tissue stimulated by inflammation, endometrial cell oxidative stress, etc. Therefore, this study included EP into the study separately according to the postoperative pathological results.

In recent years, the Human Microbiome Project (HMP) has found that about 9% of human microbiota exists in female genital tract (Margulies et al., 2022). In the past, it was believed that female genital tract microorganisms only inhabit the vagina, vulva, anus and so on. Because of the existence of the cervix and cervical mucus plug, the vagina and the upper genital tract are perfectly isolated, and the sterile state of the uterine cavity is maintained. However, more and more evidence shows that the female genital tract is an open system. From the external genitalia to the internal genitalia, there is a gradually changing microbiota continuum. The bacterial abundance from vagina to ovary decreases, while the bacterial diversity increases (Peterson et al., 2009; Cicinelli et al., 2015; Kitaya et al., 2015). It is reported that there are 10^2-10^4 fewer bacteria in the uterine cavity than in the vaginal microbiota ([[NoAuthor]]). Therefore, the uterine cavity contains a low abundance of bacterial communities, also known as low biomass microbiota. These low abundance microbiota constitute the intrauterine microecology. Many studies, including HMP, have found the importance of microorganisms and their genomes in human health and disease (Chen et al., 2017; Margulies et al., 2022). So we boldly speculate that chronic endometritis (CE), endometrial polyps (EP), and even endometrial cancer may occur when the microbiota in the uterus changes or pathogens invade. The study found that among many endometrial diseases, CE and EP are more common in patients with infertility and become the main factors affecting the outcome of assisted reproduction (Human Microbiome Project Consortium, 2012; Miles et al., 2017; Peric et al., 2019).

By using 16S ribosomal RNA(16S rRNA) sequencing technology, this study investigated and analyzed the vaginal, cervical and endometrial biopsy (EB) samples of 134 infertile patients who were ready for assisted reproductive technology (ART), and took endometrial tissue for pathological examination. The main purpose of this study is to further explore the genital tract microecological characteristics of infertile patients with CE and EP by analyzing the germs (especially the upper genital tract) of asymptomatic infertile patients, and lay a foundation for finding the pathogenesis of the disease and effective diagnosis and treatment methods.

Methods

Study design and study population selection

In this prospective study, we analyzed vaginal microbiome (VM), cervical microbiome (CM), and endometrial microbiome (EM) in infertile patients prepared for ART treatment by 16S rRNA

sequencing. At the same time, endometrial tissue was obtained during hysteroscopy for pathological examination and immunohistochemical examination, and pathological diagnosis was made according to the current CE and EP pathological diagnostic criteria, which served as the basis for grouping.

The main purpose of this study is to conduct a preliminary study on the characteristics of the genital tract microbiota of CE patients, especially the endometrial microbiota, and to study the possible pathogenesis of CE by comparing the microbiota of the genital tract in CE, EP and normal control groups.

In this study, infertile patients who are ready to receive assisted reproductive technology are selected as the research object, mainly based on the following considerations: First, according to the existing research data, the CE incidence of infertile patients is higher than that of other populations. As a prospective study, it is easier to obtain positive patients; Second, the hormone levels of patients who are ready to receive ART have been adjusted to an average level before embryo transfer, minimizing the impact of hormones on the genital tract microecology.

Study population—inclusion and exclusion criteria

Select the patients who are scheduled to undergo hysteroscopy before ART in our hospital, since October 2020 and meet the inclusion criteria, and take the genital tract microbial samples and endometrial tissue samples of the patients during hysteroscopy.

Inclusive criteria were: age>18 years and<45 years; within 3-5 days of clean menstruation (excluding abnormal vaginal bleeding); the serological tests of human immunodeficiency virus, hepatitis B, hepatitis C virus and syphilis were negative; the vaginal discharge was normal before operation; no antibiotics were taken within 30 days before operation; no sexual life within 3 days before operation; there was no vaginal irrigation or medication within 7 days before operation.

Exclusion criteria were: acute stage of various female genital tract inflammation; systemic acute inflammation; patients with autoimmune diseases; intrauterine placement of birth control ring; there are uterine cavity operators within 3 months.

Sample collection

All samples in this study were collected during hysteroscopic surgery: 1) Vaginal secretions were collected with sterile cotton swabs before sterilization and placed in sterile PBS; 2) Specimens of cervical secretion: after routine surgical disinfection, expose the cervix, place the cervical microbial sampling brush in the cervical tube and rotate it for 10 circles, collect the cervical secretion samples and put them in sterile PBS; 3) Specimens of uterine secretion: insert the special endometrial sampling brush with a sleeve into the uterine cavity, withdraw the sleeve, expose the brush head and rotate for 10 circles to collect samples of uterine secretion, push back the sleeve, take out the endometrial sampling brush, and place the sample in sterile PBS. 4) Endometrial tissue samples: after inserting the hysteroscope according to the routine operation of hysteroscopy, observe and record the condition of the endometrium. At the same time, gently scratch the uterine cavity with a small curette for one week, and collect endometrial tissue samples. After all samples are taken, samples of vaginal secretions, cervical canal secretions and uterine cavity secretions shall be immediately stored in a refrigerator at - 80 ° C and sent to the laboratory for 16S rRNA sequencing analysis. Endometrial tissue samples were placed in sterile formalin solution and sent for pathological examination.

Pathological diagnosis and grouping

The diagnosis and grouping of all enrolled patients were determined based on the pathological examination results of endometrial tissue samples. All patients with pathological diagnosis of “chronic endometritis” or “chronic inflammation of the endometrium with extensive lymphocyte infiltration”, and at least one positive item of CD38 or CD138 in immunohistochemical examination results, are included in the CE group. Patients with pathological findings of “endometrial polyps” or “endometrial polypoid hyperplasia” were included in the EP group. The other patients without abnormal pathological diagnosis were all included in the normal control group, namely NE group. According to the above grouping, the corresponding 16S rRNA sequencing results were statistically analyzed.

Sequencing

1. Extraction of genome DNA: Total genome DNA from samples was extracted using CTAB method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/μL using sterile water.

2. Amplicon Generation: 16S rRNA genes of distinct regions (16S V4/16S V3/16S V3-V4/16S V4-V5) were amplified used specific primer(e.g. 16S V4: 515F 806R, et. al) with the barcode. All PCR reactions were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 2 μM of forward and reverse primers, and about 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s. Finally 72°C for 5 min.

3. PCR Products quantification and qualification: Mix same volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. PCR products was mixed in equidensity ratios. Then, mixture PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany).

4. Library preparation and sequencing:Sequencing libraries were generated usingTruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer’s recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Data analysis

Raw data obtained from sequencing has a certain proportion of Dirty Data. In order to make the results of information analysis more accurate and reliable, the original data shall be spliced and filtered to obtain valid data (Clean Data).

Based on the effective data, OTUs (Operational Taxonomic Units) clustering and species classification analysis were carried out. According to the OTUs clustering results, on the one hand, species annotation was made for the representative sequence of each OTU to obtain the corresponding species information and the abundance distribution based on species. At the same time, OTUs were analyzed by abundance, Alpha diversity calculation, Venn map and petal map to obtain the species richness and evenness information in the sample, and the common and unique OTUs information among different samples or groups. On the other hand, multiple sequence alignment can be carried out for OTUs, and phylogenetic trees can be constructed, and further differences in community structure of different samples and groups can be obtained, which can be displayed through dimension reduction maps such as Beta diversity calculation, PCoA or PCA.

In order to further explore the differences in community structure among grouped samples, T-test, MetaStat, LEfSe and MRPP statistical analysis methods were used to test the difference significance of species composition and community results of grouped samples.

Results

Patient cohort, characteristics, and outcomes

From October 2020 to July 2021, 169 infertile patients in our department who are ready to receive IVF treatment were evaluated. Twenty nine patients were excluded from the study because they did not meet the inclusion criteria (n=29) or refused to participate (n=4). The remaining 134 people were enrolled in the study, and the composition of microbiota in genital tract of patients with different pathological types was evaluated by 16S rRNA sequencing and pathological examination. Among them, there were 29 CEs (21.6%), 20 EPs (14.9%), and 85 normal endometrium (showing normal secretory or proliferative endometrium) (63.5%). The average age of the patients was 33 years (33 ± 7.6years). There was no significant difference in general clinical data between groups.

General situation of genital tract microbiota composition

Due to the low abundance of microbiota in the endometrium, we performed a rigorous analysis of the sample data to ensure that contamination readings did not interfere with downstream analysis. The undetectable samples are excluded by comparing the samples with the samples in each run (including blank samples) and evaluating certain quality parameters.

All detectable samples were included in the analysis according to the above standards, and the overall analysis results of the samples showed that: *Lactobacillus* is the main genus in all samples. At the same time, bacterial genera (such as *Acinetobacter, Atopobium, Fusobacterium, Gardnerella, Peptostreptococcus, Staphylococcus, Streptococcus* and *Streptococcus*) and *Chlamydia* are also common. All genital tract microbial samples are grouped according to vagina, cervix and endometrium, and the relative abundance and distribution of species in each group are shown in Figure 1A. Statistical analysis shows that the abundance of the same genus in different parts is significantly different (and Figures 1B, C).

From Figures 1B, C, we can find that at the genus level, the relative abundance of *Lactobacillus, Gardnerella, Atomium* and *Streptococcus*, decreases gradually from the lower genital tract to the upper genital tract, while that of *Staphylococcus, Ralstonia* and *Acinetobacter*, including others, increases gradually. *Chlamydia* and *Fusobacterium* have high abundance in the cervix.

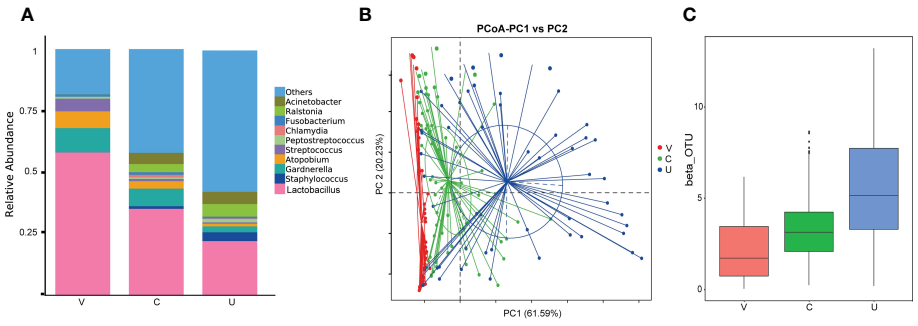

FIGURE 1
(A) Histogram of Relative Abundance Distribution of Microbes in Patient's Genital Tract. List the top ten microorganisms in relative abundance on genus level and others. Group name: V= vaginal microbiome, C=cervical microbiome, and U=endometrial microbiome. **(B, C)** Comparative analysis of microbial community composition in genital tract. PoCA shows clustering between groups of samples. Beta diversity index box chart and table visually shows significant differences between different groups. Group name: V= vaginal microbiome, C=cervical microbiome, and U=endometrial microbiome.

Taxonomic analysis of species in genital tract of CE and EP patients

At the same time, we also divided the patients into CE group, EP group and normal endometrium group(NE) according to the pathological examination results, and on the level of phylum, class, order, family, genus and species, the composition of microbiota in different parts of genital tract of patients with different pathological results was analyzed.

OTU cluster analysis

We use software to cluster the sequence data obtained at 97% similarity level to get OUT. According to the clustering results, we analyzed the common and unique OTUs among different sample groups (Table 1).

Taxonomic analysis of species

Complete the OUT clustering and annotate the OTUs sequence. According to the species annotation results, select the top 10 species with the highest abundance on each classification level (Phylum, Class, Order, Family, Genus, Species) for each grouping, and generate a histogram of species relative abundance (Figures 2A–F) . At the same time, the composition ratio of the patient's genital tract microbiota on each level is generated. Because the distribution of the microbiota at each level is highly consistent, only the composition ratio tables at the phyla level and genus level are listed in this paper (Tables 2, 3).

As can be seen from the Figure 2A, the top 10 on phyla level include *Firmicutes, Actinobacteriota, Proteobacteria, Fusobacteriota, Chlamydiae, Bacteroidota, Campylobacterota, Synergistota, Spirochaetota* and unidentified_Bacteria.

Compared with NE group, among the top five phylums in CE group, the distribution of *Firmicutes* in the whole genital tract increased significantly. On the contrary, *Actinobaciota*, which ranked second, showed a decreasing trend. The distribution of *Proteobacteria* in endometrium was significantly reduced. *Fusobacteriota* decreased in vagina and cervix, but increased in uterine cavity. *Chlamydiae* is almost not detected in the vagina and uterine cavity, but it is detected in the cervix, and the abundance of CE patients is significantly increased. However, in NP group, the distribution trend of *Firmicutes* and *Actinobaciota* in the whole genital tract was the same as that in CE group. *Proteobacteria* showed a decreasing trend in the distribution of the whole genital tract. *Fusobacteriota* increased in the whole genital tract. *Chlamydiae* was not detected in the genital tract of NP patients.

At the class level, the top 10 classes mainly come from the top 5 phylums. They are mainly Bacilli, Clostridia, Negativicutes from Firmicutes, and Actinobacteria, Coriobacteriia from Actinobacteria. Compared with NE group, the distribution change of relative abundance of microbiota in CE group and NP group was consistent with the change of phylum level.

Through statistical analysis, we also found that at the level of orders, families, genera and species, the microbial species with higher relative abundance also mainly come from the top five phyla, and the species with higher relative abundance and their proportion increase or decrease are consistent with the level of phyla and class.

Species abundance clustering heat map

In order to further clarify the difference in relative abundance of microorganisms among groups and find out the distribution characteristics of genital tract flora of CE and NP patients, according to the species annotation and abundance information of all samples on the genus level, the top 35 genera with abundance ranking are selected. According to their abundance information, they are clustered from two different levels, namely species and grouping, and drawn into a heat map, so as to more intuitively find the species that gather more or less in each group, as well as the differences between groups (Figure 3).

TABLE 1 Number of OTUs in genital tract specimens of patients in each group.

Group	Number of OTUs	Subtotal	Total
CEV	441	5979	17685
CEC	3274		
CEU	2264		
PV	316	2550	
PC	1103		
PU	1131		
NV	843	9156	
NC	5397		
NU	2916		

Group name: same as Figure 2.

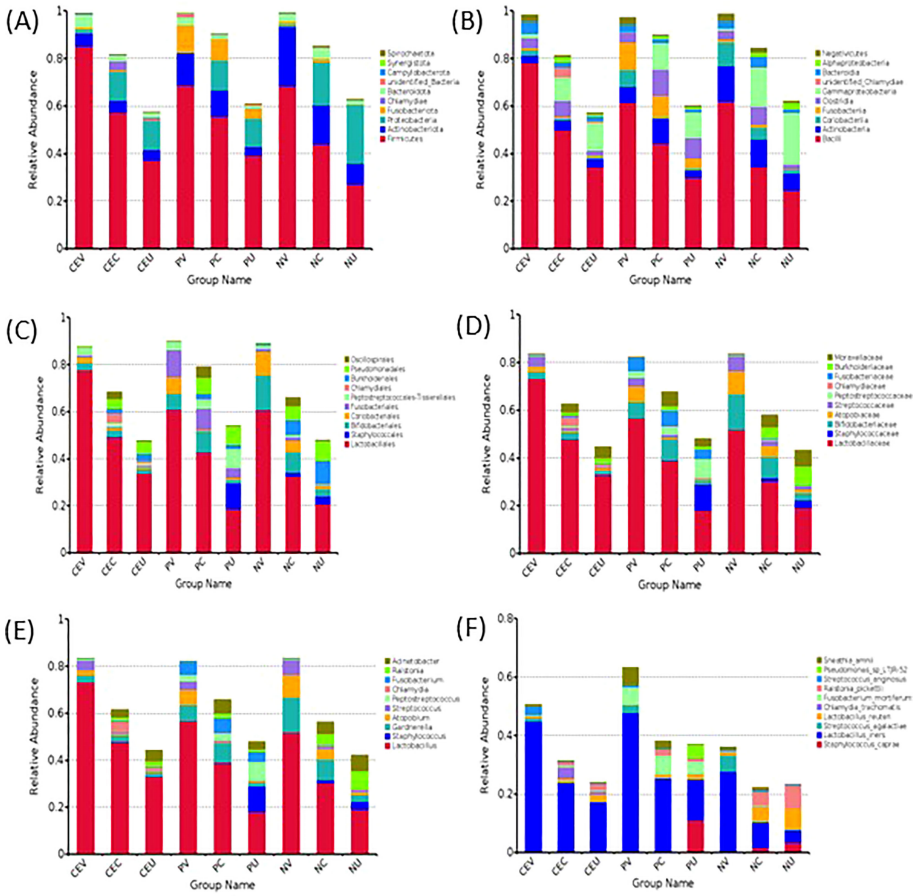

FIGURE 2
Histogram of Relative Abundance Distribution of Microbes in Patient's Genital Tract. List the top ten microorganisms in relative abundance on phyla level **(A)**, class level **(B)**, order level **(C)**, family level **(D)**, genus level **(E)** and species level **(F)** without others. Group name: CEV=CE patients' vaginal microbiome, CEC=CE patients' cervical microbiome, and CEU= CE patients' endometrial microbiome; PV=EP patients' vaginal microbiome, PC= EP patients' cervical microbiome, and PU=EP patients' endometrial microbiome; NV= vaginal microbiome in patients with normal endometrium, NC=cervical microbiome in patients with normal endometrium, and NU=endometrial microbiome in patients with normal endometrium.

TABLE 2 Composition ratio of patients' genital tract microbiota on phylum level.

Taxonomy	NV	CEV	PV	NC	CEC	PC	NU	CEU	PU
Firmicutes	0.684	0.851	0.686	0.438	0.572	0.555	0.269	0.372	0.390
Actinobacteriota	0.250	0.055	0.136	0.165	0.051	0.113	0.090	0.045	0.039
Proteobacteria	0.010	0.017	0.002	0.182	0.122	0.124	0.246	0.121	0.118
Fusobacteriota	0.012	0.008	0.116	0.014	0.007	0.090	0.002	0.005	0.043
Chlamydiae	0.000	0.000	0.000	0.001	0.039	0.001	0.000	0.010	0.000
Bacteroidota	0.032	0.054	0.032	0.045	0.023	0.017	0.015	0.019	0.012
unidentified_Bacteria	0.004	0.002	0.018	0.004	0.002	0.003	0.004	0.002	0.005
Campylobacterota	0.001	0.004	0.001	0.003	0.001	0.002	0.002	0.001	0.000
Synergistota	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000
Spirochaetota	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002

Group name: same as Figure 2.
List the top 10 microorganisms in relative abundance on phyla level.

TABLE 3 Composition ratio of patients' genital tract microbiota on genus level.

Taxonomy	NV	CEV	PV	NC	CEC	PC	NU	CEU	PU
Lactobacillus	0.519	0.732	0.567	0.301	0.477	0.388	0.190	0.330	0.181
Staphylococcus	0.001	0.000	0.000	0.016	0.003	0.003	0.034	0.001	0.110
Gardnerella	0.148	0.030	0.067	0.087	0.028	0.082	0.028	0.017	0.016
Atopobium	0.096	0.023	0.066	0.047	0.007	0.005	0.015	0.007	0.007
Streptococcus	0.063	0.041	0.036	0.010	0.008	0.006	0.007	0.004	0.002
Peptostreptococcus	0.005	0.006	0.027	0.003	0.004	0.032	0.002	0.002	0.079
Chlamydia	0.000	0.000	0.000	0.001	0.039	0.001	0.000	0.010	0.000
Fusobacterium	0.002	0.002	0.058	0.003	0.005	0.064	0.001	0.003	0.042
Ralstonia	0.000	0.000	0.000	0.046	0.013	0.019	0.077	0.023	0.012
Acinetobacter	0.000	0.000	0.000	0.051	0.033	0.059	0.068	0.046	0.031

List the top 10 microorganisms in relative abundance on genus level. Group name: same as Figure 2.

Alpha diversity analysis

In order to evaluate the differences in species richness and diversity of microbial communities in various groups, we made statistics on the alpha diversity analysis index (chao1, shannon) of different samples under the 97% consistency threshold. At the same time, analyze whether the species diversity difference between groups is significant by Wilcox rank sum test (Figures 4A, B).

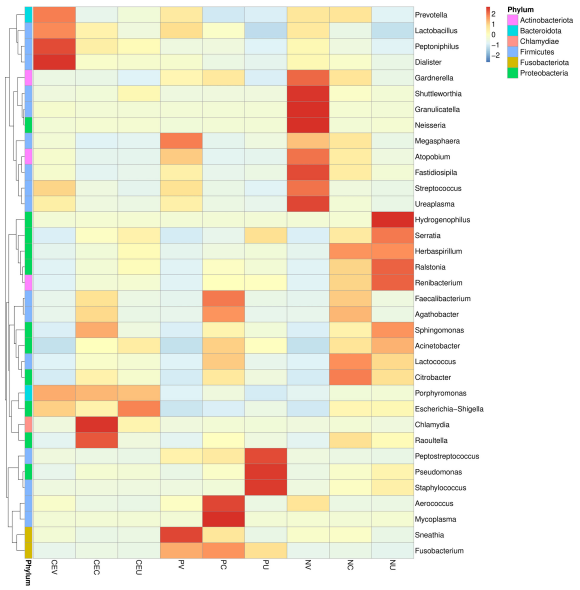

FIGURE 3
Cluster heat map of microbial species abundance in genital tract of patients in each group. In the figure, the horizontal is the sample information of each group, the right side is the species annotation information, and the left side is the species clustering tree. The color of each grid indicates the abundance of species. Group name: same as Figure 2.

Beta diversity analysis

In order to compare the difference of microbial community composition of different sample groups, we made statistics on the beta diversity analysis. And, analyze whether the species composition difference between groups is significant by Wilcox rank sum test (Figure 4C).

Discussion

Chronic endometritis (CE) is a chronic inflammatory disease with endometrial interstitial plasma cell infiltration, which is mainly caused by changes in endometrial microecology and infection of pathogens (Chen et al., 2017; Moreno and Cicinelli, 2018). And endometrial polyps (EPs) are a kind of pathological changes that protrude from the surface of endometrium, which are composed of endometrial glands and fibrotic endometrial stroma containing blood vessels (Nijkang et al., 2019). The main purpose of this study is to understand the composition and change characteristics of the microbiota in the genital tract (especially in the endometrium) of infertile patients with CE or EPs, and to explore its correlation with disease occurrence. Some diseases or organ dysfunction are characterized by the presence of potentially pathogenic microorganisms. Research based on molecular sequencing technology shows that the microbiota of female genital tract is in dynamic balance under healthy conditions, and the change of microbiota composition of genital tract may be related to a variety of gynecological diseases, such as chronic endometritis (CE), endometrial polyps (Eps), endometriosis (EMs), endometrial cancer (EC), infertility, etc (Al-Nasiry et al., 2020). From Figures 1A–C, and other results, we can easily find that the microorganisms in the genital tract show the characteristics of continuous distribution, and the species abundance in different parts of the genital tract has a significant difference, indicating that different parts of the genital tract have different microbial distribution characteristics, in other words, they have different

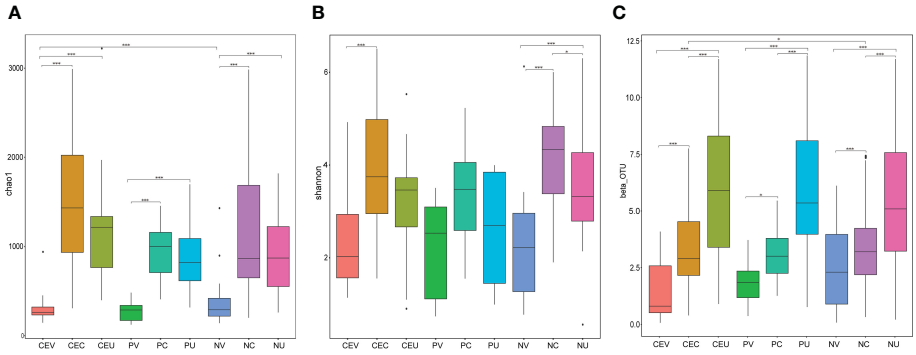

FIGURE 4
Box Chart of Chao1 **(A)**, Shannon **(B)** Index and Beta Diversity **(C)** of Genital Tract Microbial Samples of Patients in Each Group. Group name: same as Figure 2.

micro ecological environments. When local microbial status changes or pathogenic bacteria invade, corresponding dysfunction or disease will occur.

About the OTUs

In the OUT cluster analysis, we found that, according to the location, the number of species from vagina to uterine cavity increased gradually. According to disease grouping, the number of genital tract species in NE group was significantly higher than that in CE and NP groups, and the number of species in CE group was higher than that in NP group.

This result indicates that the abundance of microorganisms gradually decreases from the lower genital tract to the upper genital tract, but the number of species gradually increases. Similar findings have also been made in previous studies. Mile et al (Miles et al., 2017). evaluated the distribution of bacteria in the entire female genital tract, including the fallopian tubes and ovaries, and found that bacteria colonized the entire female genital tract, and the microbial community at each anatomical site was highly correlated. Chen et al. (2017) also found that bacterial abundance gradually decreased from lower to upper in the genital organs. Different bacterial communities were found in the cervical canal, uterus, fallopian tubes, and abdominal fluid compared to the vagina, and the bacterial composition in the endometrium was significantly different from that in the lower genital tract.

Fang et al. (2016) found that there were significant differences in the uterine microbiota between CE group patients and normal individuals, and the normal control group had a more diverse intrauterine microbiota than CE group patients. This is similar to our research results, indicating that the normal population has the most abundant microbial species in the genital tract, which is necessary to maintain a healthy microecology.

About diversity analysis

whether in Alpha diversity analysis or Beta diversity analysis, we found that there were some differences between groups among microbial samples from the same genital tract in different disease groups, but these differences were not statistically significant. It indicates that there is no significant difference between CE or NPs patients and NE patients in terms of microbial species diversity or microbiota composition. Therefore, it can be inferred that the changes of local microecology of genital tract in patients with CE or Eps are mainly due to the invasion of pathogenic bacteria and the changes of the abundance of symbiotic bacteria *in situ*, and the occurrence of disease is more closely related to the changes of the abundance of bacteria.

Changes in the composition of vaginal microbiota in patients

In a healthy state, the vaginal microbiota is in a dynamic equilibrium state dominated by *Lactobacillus*, which changes with menstrual cycle, age, pregnancy, sexual behavior, environment and other factors (Champer et al., 2018). When the composition of the lower genital tract microbiota changes, the risk of uterine cavity colonization caused by bacteria ascending increases, which can induce uterine cavity microecology disorder, and then lead to endometrial lesions. Marchenco et al (Marchenko et al., 2016). also found that the risk of vaginal bacteria colonization in patients with vaginal microecology disorder was 3.5 times higher than that in healthy women. Kovalenko et al. (2009) also proposed that *Gardnerella vaginalis* associated with BV can promote the occurrence of EPs.

From the results of species classification analysis (Figure 2, Tables 2, 3), it can be found that compared with the EN group, the CE group and NP group had significant changes in the vaginal microbiota abundance. The main manifestation was that the abundance of dominant *Lactobacillus* was significantly increased, especially in the CE group. The abundance of *Gardnerella, Atopobium and Streptococcus* in the two disease groups decreased significantly. It can be seen more intuitively from the Species isolation clustering heat map that the distribution area of the dominant and inferior vaginal bacteria in CE group and NP group is completely different from that in NE group. Therefore, it is reasonable to infer that the changes in the composition of vaginal

microbiota in patients with CE and NPs are related to the occurrence of diseases. It is possible that changes in the microbiota of the lower genital tract have led to an increased susceptibility to inflammation in the lower genital tract, increasing the possibility of certain bacteria retrograde to the upper genital tract, thereby altering the distribution of bacteria in the upper genital tract and altering local microbiota, resulting in an increased susceptibility to inflammation in the upper genital tract. Some studies this year also suggest that dysbiosis of the uterine microbiota may be one of the important promoting factors for the formation of CE and EP.

Changes in the composition of cervical microbiota in patients

In the past, few independent studies were conducted on cervical microbiota, and most of them were conducted jointly with vaginal microbiota. Relevant studies have shown that *Lactobacillus* is the dominant bacteria in the cervical microbiota of healthy women, and other common bacteria are *Prevotella, Streptococcus and Fusobacterium*, etc (Ata et al., 2019). At the same time, Chen et al (Kimura et al., 2019). further studied and found that the proportion of *Lactobacillus* in cervical microbiota is generally slightly lower than that in vaginal microbiota.

In this study, the cervix was studied as an independent group. Our study also found that the dominant microorganism in the cervix is *Lactobacillus*, and its overall abundance is lower than that in the vagina, but higher than that in the uterine cavity. In the cluster heat map, it was found that the cervical microorganisms in NP group were mainly *Lactobacillus* and *Citrobacter*, but there was no obvious aggregation compared with other species, and the abundance of each species was relatively average. However, in CE group and NP group, the species aggregation is obvious, and the dominant bacteria are significantly different from NE group. The dominant bacteria in CE group are *Chlamydia* and *Raoultella*, while the dominant bacteria in NP group are *Mycoplasma* and *Faecalibacterium*. In the OTU cluster analysis, we found that the number of microorganism species in the cervix of CE group and NE group was significantly higher than that in the uterine cavity. The analysis may be due to the influence of some microorganism species in the uterine cavity due to the deep sampling depth of the cervix.

Changes in the composition of intrauterine microbiota in patients

Previous studies found that the composition changes of the upper genital tract microbiota in CE and EPs patients were mainly due to the increase in the detection rate of vaginal bacteria (such as *Lactobacillus*) in the uterine cavity, while the related studies on the changes of the composition of the oviduct and ovary microbiota have not been reported. Cicinelli et al. (2009) showed that compared with the healthy control group (6.2%), the detection rate of *Lactobacillus* uteri in EPs and CE patients was higher (38.6% and 33.2% respectively). Fang et al. (2016) studied endometrial specimens of patients in the simple polyp (EP) group and the EP/CE group, and found that the intrauterine bacterial flora of patients in the EP/CE group was more diverse than that in the simple EP group and the healthy control group. *Lactobacillus, Gardnerella, Bifidobacterium, Streptococcus* and *Argyromonas* in endometrium of patients in EP/CE group and EP group were significantly increased, while *Pseudomonas* was significantly decreased. In addition, the proportion of *Enterobacteriaceae* and *Sphingolimus* in endometrium of patients in EP/CE group was decreased, and *Prevotella* was increased. It is suggested that there may be an increase of vaginal bacteria in the uterine cavity of EP/CE patients, and there is a difference in the uterine microbiota between EP/CE patients and normal people, but the relationship and mechanism are still uncertain.

In this study, we found that the lactobacillus abundance in CE group was significantly higher than that in NE group, but decreased in NP group. More interestingly, *Gardnerella* and *Atopobium*, which were found to have significantly increased abundance in other studies, were found to be significantly lower than the control group in this study. At present, the conclusions of studies on the composition and abundance of uterine microbiota are different, and further studies on the changes of specific microbial composition and pathogenic microorganisms in endometrium are still needed.

According to analysis, the bacteria species with obvious aggregation in NE group endometrium decreased significantly in CE group and NP group. On the contrary, the bacteria gathered in the two disease groups had lower content in NE group. In addition, it was also found that there was no obvious aggregation of microorganisms in the endometrium of CE group, and the concentration of each bacterium was relatively uniform, only *Porphyromonas* and *Escherichia* showed a certain degree of aggregation, and it was found that the concentrations of these two bacteria were also high in the genital tract under CE. In NP group, the intrauterine concentrations of *Peptostreptococcus, Pseudomonas* and *Staphylococcus* were significantly increased, and *Staphylococcus*, as a common pathogenic bacterium, only had a high abundance in the inner membrane of NP group. *Porphyromonas* and *Escherichia* increased in CE group, but decreased significantly in NP group. It is suggested that the occurrence of CE, NPs and other endometrial diseases is related to the reduction of normal flora. *Porphyromonas* and *Escherichia* are highly correlated with the occurrence of CE, while *Peptostreptococcus, Pseudomonas and Staphylococcus* are more correlated with the occurrence of NPs.

About mycoplasma and chlamydia

In our study, we found that the overall abundance of chlamydia was significantly higher than that of mycoplasma, and the highest abundance was found in the cervical region of CE patients. In other studies, the overall abundance of mycoplasma, which often occurs in other studies, was very low. Only in the cluster thermogram analysis, we found the accumulation of mycoplasma in the cervical region of NP group. We believe that this may be related to the selection of patient groups. We select all patients who have undergone the early standardized treatment of the reproductive

center and are ready to receive embryo transfer. Therefore, in the early stage of treatment, TCM students have already carried out systematic treatment for mycoplasma, so our results of mycoplasma are very low. Chlamydia only exists in the cervix and uterine cavity of CE, suggesting that Chlamydia may be related to the pathogenesis of CE.

Conclusion

Our research results show that, compared with the normal control group, the endometrial microbiota of infertile patients with chronic endometritis or endometrial polyps has indeed changed significantly in the relative abundance distribution of species, which indicates that changes in local microecology may be an important factor in the occurrence of diseases or even adverse pregnancy outcomes. The further study of endometrial microecology may provide new opportunities for further improving the diagnosis and treatment strategies of chronic endometritis.

Data availability statement

The data presented in the study are deposited in the BioProject (https://submit.ncbi.nlm.nih.gov/subs/bioproject/SUB12706297/overview) repository, accession number: BioProject ID: PRJNA932472.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Shanghai Tongji Hospital (SBKT-2022-036). The patients/participants provided their written informed consent to participate in this study.

Author contributions

JL and LZ designed and carried out the study. YY and XJ collected samples and clinical data. ML and QZ conducted DNA extraction and PCR experiments. TL and ZH assisted in 16S rRNA sequencing. JL, QL, and XT analyzed data and wrote papers. LZ provides an important intellectual content revision for this article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Al-Nasiry, S., Ambrosino, E., Schlaepfer, M., Morré, S. A., Wieten, L., Voncken, J. W., et al. (2020). The interplay between reproductive tract microbiota and immunological system in human reproduction. *Front. Immunol.* 11. doi: 10.3389/fimmu.2020.00378

Ata, B., Yildiz, S., Turkgeldi, E., Pérez Brocal, V., Dinleyici, E. C., Moya, A., et al. (2019). The endobiota Study:Comparison of Vaginal,Cervical and gut mierobiota between women with stage 3/4 endometriosis and health controls. *Sci. Rep.* 9 (1), 2204. doi: 10.1038/s41598-019-39700-6

AAGL Advancing Minimally Invasive Gynecology Worldwide. (2012). AAGL practice report: practice guidelines for the diagnosis and management of endometrial polyps. *J. Minim Invasive Gynecol* 19 (1), 3–10. doi: 10.1016/j.jmig,2011.09.003

Champer, M., Wong, A. M., Champer, J., Brito, I. L., Messer, P. W., Hou, J. Y., et al. (2018). The role of the vaginal microbiome in gynaecological cancer. *BJOG* 125 (3), 309–315. doi: 10.1111/1471-0528.14631

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., et al. (2017). The microbiota continuum along the female reproductive tract and its relation to uterinerelated diseases. *Nat. Commun.* 8 (1), 875. doi: 10.1038/s41467-017-00901-0

Cicinelli, E., De Ziegler, D., Nicoletti, R., Tinelli, R., and De Vito, D. (2009). Poor reliability of vaginal and endocervical cultures for evaluating microbiology of endometrial cavity in women wiyh chronic endometritis. *Gynecol Obstet Invest.* 68 (2), 108–115. doi: 10.1159/000223819

Cicinelli, E., Matteo, M., Tinelli, R., Lepera, A., Alfonso, R., Indraccolo, U., et al. (2015). Prevalence of chronic endometritis in repeated unexplained implantation failure and the IVF success rate after antibiotic therapy. *Hum. Reproduction.* 30 (2), 323–330. doi: 10.1093/humrep/deu292

Fang, R. L., Chen, L. X., Shu, W. S., Yao, S. Z., Wang, S. W., and Chen, Y. Q. (2016). Barcoded sequencing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. *Am. J. Transl. Res.* 8 (3), 1581–1592.

Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486 (7402), 207–214. doi: 10.1038/nature11234

Kimura, F., Takebayashi, A., Ishida, M., Nakamura, A., Kitazawa, J., Morimune, A., et al. (2019). Review: chronic endometritis and its effect on reproduction. *J. Obstet Gynaecol Res.* 45 (5), 951–960. doi: 10.1111/jog.13937

Kitaya, K., Matsubayashi, H., Yamaguchi, K., Nishiyama, R., Takaya, Y., Ishikawa, T., et al. (2015). Chronic endometritis: potential cause of infertility and obstetric and neonatal complications. *Am. J. Reprod. Immunol.* 75 (1), 13–22. doi: 10.1111/aji.12438

Kovalenko, V. L., Voropaeva, E. E., Kozachkov, E. L., and Kozachkova, E. A. (2009). Endometrial pathomorphology in bacterial vaginosis associated with chronic endometritis. *Arkh Patol* 70 (2), 6–8.

Marchenko, L. A., Chernukha, G. E., Yakushevskaya, O. V., Gombolevskaya, N. A., Muravieva, V. V., Priputnevich, T. V., et al. (2016). Clinical and microbiological aspects of cho\ronic endomertritis in women of reproductive age. *Antibiot Khimioter* 61 (9/10), 44–51.

Margulies, S. L., Flores, V., Parkash, V., and Pal, L. (2022). Chronic endometritis: a prevalent yet poorly understood entity. *Int. J. Gynecol Obstet.* 158, 194–200. doi: 10.1002/ijgo.13962

Miles, S. M., Hardy, B. L., and Merrell, D. S. (2017). Investigation of the microbiota of the reproductive tract in women undergoing a total hysterectomy and bilateral salpingo-oopherectomy. *Fertil Steril.* 107 (3), 813–820.e1. doi: 10.1016/j.fertnstert.2016.11.028

Moreno, I., Cicinelli, E., Garcia-Grau, I., Gonzalez-Monfort, M., Bau, D., and Vilella, F. (2018). Garcia-Grau i,et al.The diagnosis of chronic endometritis in infertile asymptomatic women:a comparative study of histology. *Am. J. Obstrtrics Gynecol* 218 (6), 602.el–602.e16. doi: 10.1016/j.ajog.2018.02.012

Nijkang, N. P., Anderson, L., Markham, R., and Manconi, F. (2019). Endometrial polyps: pathogenesis, sequelae and treatment. *SAGE Open Med.* 7, 2050312119848247. doi: 10.1177/2050312119848247

Peric, A., Weiss, J., Vulliemoz, N., Baud, D., and Stojanov, M. (2019). Bacterial colonization of the female upper genital tract. *Int. J. Mol. Sci.* 20 (14). doi: 10.3390/ijms20143405

Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J. A., et al. (2009). The NIH human microbiome project. *Genome Res.* 19 (12), 2317–2323. doi: 10.1101/gr.096651.109

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EDITED BY
Zhangran Chen,
Shenzhen Wedge Microbiology Research Co. Ltd, China

REVIEWED BY
António Machado,
Universidad San Francisco de Quito, Ecuador
Sheryl S. Justice,
The Ohio State University, United States

*CORRESPONDENCE
Changzheng Yuan
✉ Chy478@zju.edu.cn
Liying Sun
✉ sunliying@zju.edu.cn

†These authors have contributed equally to this work and share first authorship

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Patterns of pediatric and adolescent female genital inflammation in China: an eight-year retrospective study of 49,175 patients in China

Huihui Gao[1†], Yuchen Zhang[2†], Yanzheng Pan[2], Mengjia Zhao[2], Ye Qi[2], Mingming Zhou[3], Symphorosa S. C. Chan[4], Siyi Huang[2], Peige Song[2], Kun Tang[5], Liying Sun[1*] and Changzheng Yuan[1,2*]

[1]Department of Pediatrics and Adolescent Gynecology, Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, Hangzhou, China, [2]School of Public Health, Zhejiang University School of Medicine, Hangzhou, China, [3]Department of Clinical Laboratory, Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, Hangzhou, China, [4]Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Shatin, China, [5]Vanke School of Public Health, Tsinghua University, Beijing, China

Background: Genital inflammation is one of the most frequent clinical complaints among girls, which was easily overlooked by the general public. This study aimed to investigate the patterns and epidemiological characteristics of pediatric and adolescent female genital inflammation in China.

Methods: A retrospective observational study (2011 to 2018) was conducted among all female patients under the age of 0–18 years at the Department of Pediatric and Adolescent Gynecology of The Children's Hospital, Zhejiang University School of Medicine. Data were collected from the electronic medical records. The abnormal vaginal discharge of patient was collected for microbiological investigation by bacterial and fungal culture. Descriptive analysis was conducted to evaluate the genital inflammation pattern and epidemiological characteristics, including age, season, and type of infected pathogens.

Results: A total of 49,175 patients met the eligibility criteria of genital inflammation and 16,320 patients later came to the hospital for follow-up over the study period. The number of first-visit increased gradually from 3,769 in 2011 to 10,155 in 2018. The peak age of the first visit was 0–6 years old. Non-specific vulvovaginitis, lichen sclerosis, and labial adhesion were the top three genital inflammation. Among the top five potential common pathogens of vaginal infection, the prevalence of *Haemophilus influenzae* cases was the highest (31.42%, 203/646), followed by *Streptococcus pyogenes* (27.74%, 176/646), *Candida albicans* (14.09%, 91/646), *Escherichia coli* (8.51%, 55/646), and *Staphylococcus aureus* (6.35%, 41/636). The specific disease categories and pathogens of genital inflammation vary by age groups and season.

Conclusion: Our study summarizes the pattern of pediatric and adolescent female genital inflammation over an 8-year period in China, emphasizing the need for more public awareness, healthcare services and research in this field.

KEYWORDS

pediatric and adolescent gynecology, girls, genital inflammation, disease pattern, vulnerable population, pathogens

Introduction

Female genital inflammation is manifested by abnormal vaginal discharge, odor, itching, or discomfort and affects up to 75% of girls and women during their lifetime (1). It has been reported that genital inflammation is the most common gynecological disorder among pediatric and adolescent females (2, 3). However, as a frequent complaint among girls, genital inflammation is easily overlooked by the general public. Previous evidence showed genital inflammation in adults may lead to adverse outcomes, such as endometriosis, reproductive disorders, and even gynecologic malignancies (4, 5), which has received attention in adult women. Moreover, untreated and recurrent problems contribute to a severe disease burden such as increasing the incidence of HIV and female infertility (6, 7). This problem among girls influences future reproductive health and frequently causes great anxiety in children and their parents. Especially, for girls aged 0–18 who were the "future mothers," their reproductive and psychological health deserves the attention of public health and clinical staff.

The patterns and characteristics of genital inflammation among girls differ from adults, due to the significant differences in hormone levels, lifestyle habits, and sexual behavior. For example, sexually transmitted infections, which are more common in adult females, are rare in adolescent females and young females in China (8).These unique characteristics indicate that this population has been neglected and needs more research to reveal the patterns and epidemiological characteristics and understand how girls have been impacted by genital inflammation. The information can reduce misdiagnosis and inappropriate treatment. There is substantial room to improve sexual and reproductive health (SRH) services (9).

Recently, several studies have focused on genital inflammation while most of them were mainly conducted in western countries with small sample sizes (10, 11). Therefore, our study performed a retrospective analysis of data from pediatric gynecology outpatients from the Children's Hospital of Zhejiang University School of Medicine for eight consecutive years to understand the characteristics and distribution of genital inflammation in Chinese girls. This study aims to increase the understanding of genital inflammation among girls in both local and global contexts as well as provide evidence for the subsequent development of SRH services for young girls.

Methods

Study design

We assessed the data of all outpatients aged 0–18 treated in the Pediatric and Adolescent Gynecology (PAG) Department in The Children's Hospital, Zhejiang University School of Medicine, from January 2011 to December 2018. Based on the hospital outpatient service system, all electronic medical records (EMRs) of our outpatients with genital inflammation were extracted. EMRs of outpatients consisted of the basic demographic information of patients, the history of present illness, physical examination, and auxiliary examination, recorded the details of the clinician's inquiry and observation of the patients' statuses. The research roadmap was showed in Supplementary Figure 1. The study was approved by the Human Subjects Committees of the Children's Hospital, Zhejiang University School of Medicine (approval number 2019-IBR-103).

Study population

The inclusion criteria for the study were as follows: ① outpatients at the PAG clinic who were under the age of 18 between 2011 and 2018; ② Girls who were diagnosed as non-specific vulvovaginitis, bacterial vulvovaginitis, vulvovaginal candidiasis (VVC), sexually transmitted infections (STIs), labial adhesion, lichen sclerosis, vulvar abscess, and vaginal foreign body.

Exclusion criteria: ① Patients with congenital immune deficiency or diabetes; ② History of sexual life or sexual violence; ③ "Diagnosis time," "first diagnosis" and other items missing If one of the above conditions is satisfied, the patient is excluded.

Identification of microorganisms

The abnormal vaginal discharges of participants were collected according to our previous study (12).The patients were examined in lithotomy position. The labia were separated, and a swab was taken from the introitus or, if possible, the lower third of the vagina. All swabs were kept in sterile tubes before being inoculated on plates in 1 h. Culture plates included blood agar plate (Columbia base agar containing 5% defibrinated sheep blood, BioMerieux, France), Haemophilus selective medium plate (BioMerieux, France), and Neisseria gonorrhoea selective medium plate (BioMerieux, France), Sabouraud's Medium with triphenyltetrazolium chloride (triphenyltetrazolium chloride, TTC; Bosai, China).

The blood agar plates and Haemophilus-selective medium plates were cultured in an incubator containing 5–8% carbon dioxide for 16–24 h. The N. gonorrhoea−selective plates were cultured in an air incubator for 48 h. Regarding fungal culture, samples were applied to Sabouraud's Medium with TTC were cultured in an air incubator for 24–72 h at 37°C. Bacteria or fungi grown in the pure culture or as the dominant organism were classified as pathogens and were identified further. Organisms were identified with Gram positive identification card, Gram-negative identification card, or Neisseria−Haemophilus identification card in an automatic bacteria identification system (Vitek, BioMerieux, France) after Gram staining, catalase test, and oxidase test. *Hemolytic streptococci* were grouped by rapid latex test (BioMerieux, France), and *Streptococcus pneumoniae* isolates were also tested with optochin discs (Oxoid, England).

Assessment of genital inflammation

In the study, genital inflammation in the PAG department was assessed based on the first diagnosis among patients at their first clinical visit. Referring to recent literature and textbook (8, 13–15), all newly diagnosed patients were categorized into eight major diagnoses: non-specific vulvovaginitis, bacterial vulvovaginitis, VVC, STIs, labial adhesion, lichen sclerosis, vulvar abscess, and vaginal foreign body. Patients with abnormal vaginal discharge were investigated pathogenic test by bacterial or fungal culture. The pathogenic results were also collected from patients with vaginal infections between 2015 and 2018

and divided into three categories: bacterial vulvovaginitis, VVC, and STIs. Aerobic vaginitis (AV), bacterial vaginosis (BV) belonged to bacterial vulvovaginitis.

Covariates

Information on covariates, including age, date of diagnosis, date of birth, date of visit and residency, first diagnosis, visiting status (first visit or subsequent visit), and pathogen detection, were extracted from patients' EMR. The demographic characteristics were also collected. Visit year, season, and month were derived from the date of diagnosis. Age was calculated based on the time interval between the date of birth and the date of diagnosis. Based on the WHO definition of children and adolescents, we divided girls aged 0–18 into three groups: children (pediatric,0–6 years old), pre-adolescence (prepuberty, 7–9 years old), and late adolescence (late puberty, 10–18 years old). In this study, genital inflammation in the PAG department was assessed based on the first diagnosis at their first clinical visit. When patients have more severe symptoms or recurrence symptoms, they visited the PAG department for follow-up consulting. The interval time for multi-visit patients was the number of days between the date of the first visit and the last visit; the average revisits time interval for repeat patients is the total interval divided by the number of follow-up visits. One individual extracted all EMR information. The data was then independently reviewed by a second and third individual.

Statistical analysis

Descriptive analysis was conducted to evaluate the pattern of genital inflammation in children and teenagers by age, year, and season. Continuous variables were expressed as mean and standard deviation (SD), median, and quartile (Q1, Q3) according to whether the data were normally distributed. Categorical variables were presented as the number of cases and the percentage. The χ^2 test was used to detect whether there were significant statistical differences in the distribution of outpatients by three different age groups under some basic demographic characteristics. The Kruskal Wallis test was applied to data with skewed distributions across multiple age groups such as interval time. The comparison of differences in the patterns including genital inflammation issues and pathogens across age group, year, and season were evaluated using the $R \times C$ χ^2 test of the composition ratio of multiple samples and Fisher's exact probability method when the $R \times C$ χ^2 test was not the most effective analysis. p value < 0.05 was considered statistically significant. All analyses were conducted using SAS software, version 9.4 (SAS Institute Inc., Cary, NC) and R (version 4.2.3). All graphs were drawn using GraphPad Prism 8.4.3.

Results

Basic characteristics of the study population

A total of 49,175 patients with genital inflammation visited the PAG department over 8 years, and the total number of visits was 76,807. During the study period, the number of PAG patients that attended for the first visit increased gradually from 3,769 in 2011 to 10,155 in 2018. Table 1 shows the basic characteristics of patients at the first clinical visit during the study period. Patients' ages ranged from 0 to 18 with a mean ± SD of 5.03 ± 3.16 years old; 68.74% of the participants were aged 0–6 years, 22.23% were aged 7–9 years, and 9.03% were aged 10–18 years. Patients came from Zhejiang province in 94.21% of cases, with 62.05% hailing from Hangzhou, where the hospital was situated. 16,087 patients had two or more visits within 8 years. The average number of follow-up visits for them was 1.72, and the median interval between each follow-up visit was 24 days.

Genital inflammation patterns overall and by age groups

The patterns of eight categories of genital inflammation by age group were shown in Table 2. There were significant differences in the age distribution of patients with the eight subgroups of genital inflammation patterns ($p < 0.05$). In general, non-specific vulvovaginitis accounted for 61.51% (30,247/49,175) of the first visit, followed by lichen sclerosis (9,721, 19.77%) and labial adhesion (4,260, 8.66%), bacterial vulvovaginitis (3,750, 7.63%), VVC (713, 1.45%), vaginal foreign body (331, 0.67%), vulvar abscess (102, 0.21%), STIs (51, 0.10%), respectively. The median age for various subtypes of patients with genital inflammation can be found in Figure 1A. The vaginal infections include bacterial vulvovaginitis, VVC and STIs according to the etiology of vaginal secretions, of which bacteria had the highest number of patients ($n = 3,750$) with a median onset age of 7(Q1 = 5, Q3 = 9). The next was VVC with a median age of 9(Q1 = 6, Q3 = 11), and the last was STIs with a median age of 5. Children 0–6 years old had a greater frequency of non-specific vulvovaginitis, with a median onset age of 5. With a median onset age of 1 and 4, lichen sclerosis and labial adhesion were most prevalent in children aged 0 to 6. Before age 9, the vaginal foreign body was often observed in girls. Children aged 0–6- and 10–18-years old adolescent females both had a reasonably high prevalence of vulvar abscess, with proportions of 49.02 and 30.39%, respectively.

Genital inflammation patterns by year and season

Between 2011 and 2018, the number of visits in the latter 4 years was nearly twice as high as in the first 4 years (Table 1). Of the 49,175 patients with genital inflammation, non-specific vulvovaginitis was the most common problem from 2011 to 2018 (Table 2), with the highest percentage of 81.17% in 2014 (Figure 1B). From 2011 to 2013, bacterial vulvovaginitis and VVC had high rates of vaginal infections (roughly 25 and 5%, respectively) but the proportion has declined since 2014. The number of STI cases was low, and the percentage fluctuated little from year to year, consistently not exceeding 1%. The prevalence of labial adhesion had largely been on an upward trend over the last 8 years, with the proportion of the genital inflammation pattern exceeding 10% after 2017. The percentage of lichen sclerosis was above 20% in 2015 and after and reached its highest percentage of 42.07% in 2016. The composition ratio of these eight disease subtypes varies across years in the overall ($p < 0.05$, Figure 1B).

TABLE 1 Basic characteristics of pediatric and adolescent gynecology outpatients with genital inflammation at first visits, overall and by age group.

Characteristics	Overall (*N* = 49,175)	Age 0–6 (*N* = 33,804)	Age 7–9 (*N* = 10,931)	Age 10–18 (*N* = 4,440)	*p**
Age in years, mean (SD)	5.03 (3.16)	3.32 (1.90)	7.82 (0.79)	11.20 (1.45)	
Follow-up times, *N* (%)[#]					<0.0001
1	10,144 (63.06)	6,721 (66.92)	2,489 (58.40)	934 (52.41)	
2	3,370 (20.95)	2,050 (20.41)	917 (21.52)	403 (22.62)	
≥3	2,573 (15.99)	1,272 (12.67)	856 (20.08)	445 (24.97)	
Interval time, days, median (Q_1, Q_3)[#]					
First and last visit	36 (7,295)	29 (7,223)	56 (7,419)	60 (11,479)	<0.0001
Visit-to-visit	24 (7,156)	21 (7,130)	32 (7,208)	31 (8,197)	<0.0001
Year					<0.0001
2011–2014	18,217 (37.05)	12,149 (35.94)	4,236 (38.75)	1,832 (41.26)	
2015–2018	30,958 (62.95)	21,655 (64.06)	6,695 (61.25)	2,608 (58.74)	
Zhejiang province[##]	28,820 (94.21)	20,274 (94.80)	6,082 (92.63)	2,464 (93.37)	
Hangzhou city[###]	17,838 (62.08)	13,147 (64.99)	3,380 (55.74)	1,311 (53.38)	
Season					<0.0001
Spring	8,895 (18.09)	5,681 (16.81)	2,192 (20.05)	1,022 (23.02)	
Summer	12,611 (25.64)	8,961 (26.50)	2,648 (24.22)	1,002 (22.57)	
Fall	14,805 (30.11)	9,457 (27.98)	3,762 (34.42)	1,586 (35.72)	
Winter	12,864 (26.16)	9,705 (28.71)	2,329 (21.31)	830 (18.69)	

The number of outpatients with follow-up is 16,320. *p** values are derived by χ^2 test, except for interval time, where the Kruskal Wallis test was used.
[#]The number of outpatients with follow-up is 16,087.
[##]There is a missing value, and the missing value is 18,584.
[###]The missing value for patient analysis in Zhejiang cities is 70.

TABLE 2 The proportion of specific genital inflammation issues in PAG department and by age group ($p < 0.0001$).

Subtypes, *N* (%)	Overall*	Age 0–6[#]	Age 7–9[#]	Age 10–18[#]	$p(\chi^2)$
Non-specific vulvovaginitis	30,247 (61.51)	19,810 (65.49)	7,543 (24.94)	2,894 (9.57)	<0.0001(5143.5084)
Lichen sclerosis	9,721 (19.77)	7,775 (79.98)	1,481 (15.24)	465 (4.78)	
Labial adhesion	4,260 (8.66)	4,207 (98.76)	37 (0.87)	16 (0.37)	
Bacterial vulvovaginitis	3,750 (7.63)	1,518 (40.48)	1,517 (40.45)	715 (19.07)	
Vulvovaginal candidiasis	713 (1.45)	220 (30.86)	194 (27.21)	299 (41.94)	
Vaginal foreign body	331 (0.67)	188 (56.80)	124 (37.46)	19 (5.74)	
Vulvar abscess	102 (0.21)	50 (49.02)	21 (20.59)	31 (30.39)	
STIs	51 (0.10)	36 (70.59)	14 (27.45)	1 (1.96)	

STIs: Sexually transmitted infections. $p < 0.01$ means there were significant differences in the distribution of outpatients under three different age groups and this difference was seen in at least two of the eight subtypes mentioned above. R × C χ^2 test was used for comparison of the differences in age composition ratios of the eight subtypes.
*Percentage (%) is the proportion of each individual subtype in the genital inflammation pattern.
[#]Percentage (%) is the proportion of each individual subtype under different age groups.

The cumulative number of patients in the past 8 years in summer, fall, and winter was more than 10,000, and that in spring was less than 10,000 (Table 1). Figure 1C shows that the proportions of people with non-specific vulvovaginitis and lichen sclerosis were roughly evenly distributed across four seasons. Bacterial vulvovaginitis was more common in spring (839, 9.39%) and summer (1,086, 8.61%).VVC (305, 2.06%), STIs (20, 0.14%), vaginal foreign bodies (122, 0.82%), and vulvar abscesses (1,214, 8.20%) were more common in the fall. The labial adhesion increased in the winter (1,338, 10.40%) but bacterial vulvovaginitis significantly reduced in the winter (614, 4.77%). The composition ratios of these eight disease subtypes in seasons presented significant differences overall during the 8 years ($p < 0.05$, Figure 1C).

The distribution of pathogens in patients with vaginal infections

Between 2015 and 2018, 666 cases completed pathogenic testing and detailed basic demographic information and 646 examinations were included in the statistical analysis. From the 4 years of data available on the top 10 potential common microbial pathogens, the

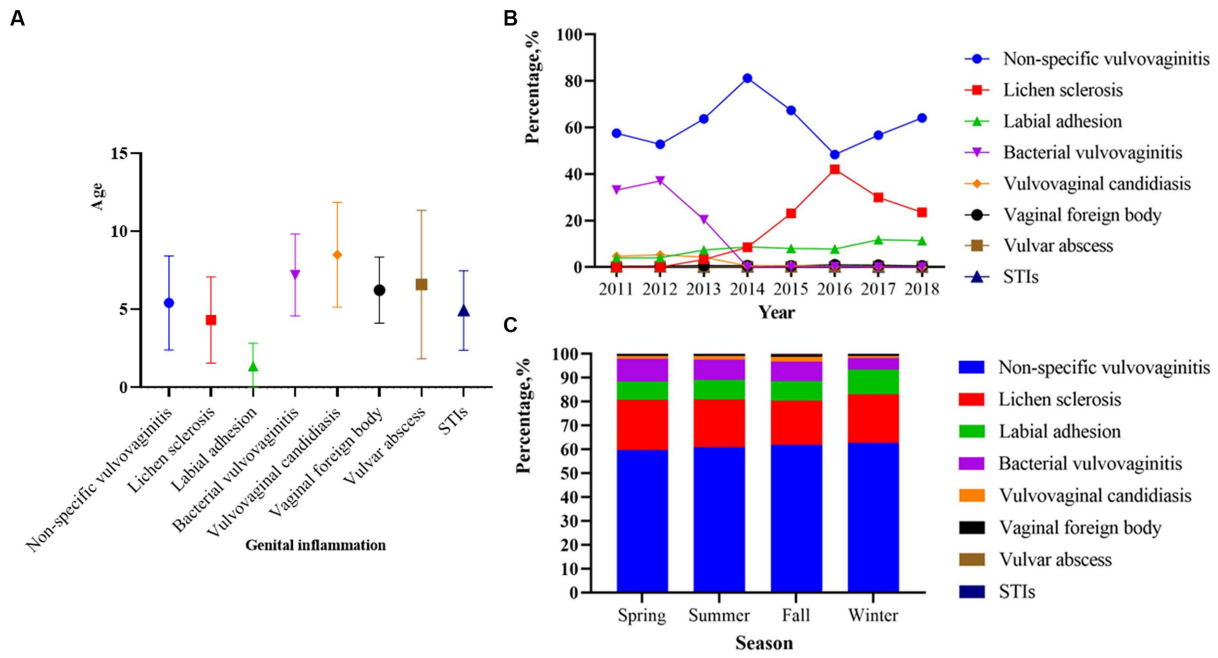

FIGURE 1
Age, year and season distribution of genital inflammation patterns among 49,175 outpatients from 2011 to 018 ($p < 0.01$). **(A)** Median age of onset corresponding to different genital inflammation patterns. **(B)** The percentage of genital inflammation patterns across different years ($p < 0.01$). **(C)** The percentage of genital inflammation patterns across different seasons ($p < 0.01$). $p < 0.0001$ in **(B)** represents a significant difference in the overall composition ratio of the above eight genital inflammation issues over the 8-year period. R × C χ^2 test of composition ratios was used for comparing whether there was a difference in the composition ratio of eight issues in these years. $p < 0.0001$ in **(C)** represents a significant difference in the overall composition ratio of the above eight genital inflammation issues over four seasons. R × C χ^2 test of composition ratios was used for comparing whether there was a difference in the composition ratio of eight issues in four seasons.

prevalence of *Haemophilus influenzae* cases was the highest (203, 31.42%), followed by *Streptococcus pyogenes* (176, 27.74%), *Candida albicans* (91, 14.09%), *Escherichia coli* (*E. coli*; 55, 8.51%), *Staphylococcus aureus* (41, 6.35%), *Klebsiella pneumoniae* (12, 1.86%), *Streptococcus agalactiae* (8, 1.24%), *Streptococcus pneumoniae* (6, 0.93%), *Haemophilus parainfluenzae* (5, 0.77%), *Neisseria gonorrhoeae* (4, 0.62%). And the age distribution of patients within the 10 pathogen classifications was statistically different ($p < 0.05$, Table 3). *Haemophilus influenzae* represented the highest proportion of cases in the group aged 0–6 (152, 74.88%). In groups of 0–6 and 7–9, other pathogens with significant age peaks are *Streptococcus pyogenes* (90, 51.14% and 81, 46.02%), *Escherichia coli* (32, 58.18% and 21, 38.18%), *Staphylococcus aureus* (18, 43.90% and 21, 51.22%), and *Klebsiella pneumoniae* (4, 33.33% and 6, 50.00%). These pathogens above are uncommon in adolescents aged 10–18. However, *Candida albicans* are highly prevalent in girls aged 10–18 years (70, 76.92%), followed by girls aged 7–9 years (21, 23.08%).

The year and season distribution of patients also differed significantly within the 10 pathogen classifications ($p < 0.05$, Figures 2A,B). From 2015 to 2018, *Haemophilus influenza*, *Streptococcus pyogenes* and *Candida albicans* were the main pathogens, and there was a gradual increase in the proportion. *Escherichia coli* showed a decreasing trend, and the other two pathogens accounted for a relatively stable proportion (Figure 2A). In terms of seasonal distribution (Figure 2B), *Haemophilus influenza* was the most common bacterial infection in summer at 42.53%. *Streptococcus pyogenes* were the most common bacterial infections in winter and spring, accounting for 36.59 and 34.04%, respectively. *Escherichia coli* and *Klebsiella pneumonia* were often seen in fall accounting for 13.17 and 6.59%, respectively. *Staphylococcus aureus* was rare in spring and evenly distributed in other seasons. *Candida albicans* were prevalent in all seasons, and data showed that the highest detection rate was in winter at 18.90%.

Discussion

In the current study, we explored the patterns and epidemiological characteristics of genital inflammation among girls through an eight-year retrospective study of 49,175 patients. To best of our knowledge, this is the first large-sample and long-time-span retrospective observational study in China. Moreover, the consultation of genital inflammation has been increasing year by year in recent years, which indicated that genital inflammation among girls aged less than 18 might entail more attention from doctors, families, and society. The patterns of genital inflammation between genital inflammation in 0–18 girls and adults are significantly different. In adults, vulvovaginitis, cervicitis, and pelvic inflammation are common problems. Vulvovaginitis among adults is usually caused by bacterial, fungal, mycoplasma, chlamydia, and human papillomavirus (16).The most widespread issue among children and adolescents is non-specific vulvovaginitis (30,247, 61.51%). Lichen sclerosis, labial adhesion, bacterial vulvovaginitis, vulvovaginal candidiasis, vaginal foreign body, vulvar abscess, and STIs are in order, depending on the prevalence. Our result is consistent with western previous reviews

TABLE 3 Age characteristic of patients with genital inflammation of top10 pathogens, overall and by age groups from 2015 to 2018 ($p < 0.0001$).

No	Pathogens, *N*(%)	Overall*	Age 0–6#	Age 7–9#	Age 10–18#	*p*
1	*Haemophilus influenzae*	203(31.42)	152(74.88)	49(24.14)	2(0.99)	0.0004998
2	*Streptococcus pyogenes*	176(27.74)	90(51.14)	81(46.02)	5(2.84)	
3	*Candida albicans*	91(14.09)	0	21(23.08)	70(76.92)	
4	*Escherichia coli*	55(8.51)	32(58.18)	21(38.18)	2(3.64)	
5	*Staphylococcus aureus*	41(6.35)	18(43.90)	21(51.22)	2(4.88)	
6	*Klebsiella pneumoniae*	12(1.86)	4(33.33)	6(50.00)	2(16.67)	
7	*Streptococcus agalactiae*	8(1.24)	2(25.00)	3(37.50)	3(37.50)	
8	*Streptococcus pneumoniae*	6(0.93)	5(83.33)	1(16.67)	0	
9	*Haemophilus parainfluenzae*	5(0.77)	3(60.00)	1(20.00)	1(20.00)	
10	Neisseria gonorrhoeae	4(0.62)	3(75.00)	1(25.00)	0	
	Others	45(6.97)	21(46.67)	16(35.56)	8(17.78)	

The number of cases of the top 10 pathogens mentioned above was 646, and the number of cases of other pathogens was 45, which was not reflected in the table. $p < 0.01$ means the age distribution of the above pathogens was statistically different in at least two groups of patients with different pathogens. R × C fisher's exact test was used for comparison of the differences in age composition ratios of the 10 pathogens.

*Percentage (%) is the proportion of each bacteria pathogen among the 10 major pathogens.

#Percentage (%) is the proportion of each kind of pathogen under different age groups.

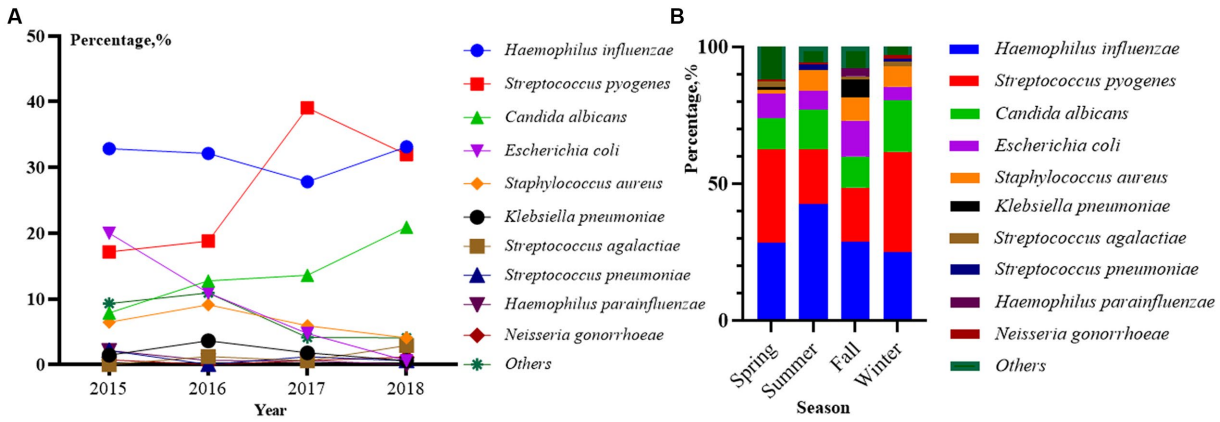

FIGURE 2
Distribution of top 10 pathogens among 646 outpatients by year **(A)** and season **(B)** from 2015 to 2018 ($p < 0.01$). $p < 0.01$ in **(A)** represents a significant difference in the overall composition ratio of the above 10 pathogens over 4 years. R × C fisher's exact test was used for comparing whether there was a difference in the composition ratio of 10 pathogens in 4 years (Others were not included). $p < 0.01$ in **(B)** represents a significant difference in the overall composition ratio of the above 10 pathogens over four seasons. R × C fisher's exact test was used for comparing whether there was a difference in the composition ratio of 10 pathogens in four seasons (Others were not included).

about genital infection in children and adolescents and completely different from the spectrum of adult diseases (8, 17). Secondly, adult genital inflammation was closely associated with a history of sexual intercourse, abortion, and unclean cervical manipulation, with a higher proportion of endogenous infections than exogenous infections. STIs are relatively common diseases in adults (18). For children and adolescents, the presentence of first-visit (0.1%) for STIs was extremely low (51, 0.1%) in the current study. The history of our patients with STIs showed they did not have sexual life or sexual abuse. Some of their parents or grandparents have a similar infection. Some of them have been to the public bathroom or swimming pool. The possible reason for preschool girls with STIs is not due to sexual transmission in our study, which is quite different from the epidemic characteristics of girls in other countries. In the United States, approximately 20 million new STIs occur, half of the cases among adolescents aged 15–24 years (19). In all, our study described the whole picture of epidemiological characteristics and categories of girls' genital inflammation in China, not only including vulvovaginitis caused by bacteria or fungi, but also vaginal foreign body and other factors.

The age distribution characteristic of genital inflammation was mainly concentrated in preschool girls in the current study. Hypoestrogenism, the anatomical proximity of the rectum, and delicate vulvar skin and vaginal mucosa are the physical features of preschool girls (13). Other different risk factors for children should also be considered, such as the long-time diaper use, improper urination habits, chemical irritants, poor hygiene, higher consumption of energy-dense, and high-glycemic-index foods (2, 20). Allergic problem is also a possible risk factor for the age distribution of genital inflammation. A multicenter study indicated that atopic dermatitis was observed most

in the1-4 years' groups (21). Previous studies indicated a strong association between allergic disease and lichen sclerosis (22, 23). Considering allergic factors are risk factors of lichen sclerosis, allergic problems should receive more attention in preschool girls. What's more, psychological factor should also consider. Preschool girls in China were usually reared by mother or grandparents during 0–3 years old and went to the kindergarten during 3–6 years old. The previous study found that 3–6 years old children had high scores of separation anxiety disorder, which may attribute to their transition from family to kindergarten (24). We found a kind of special genital inflammation, a vaginal foreign body, was also common during this period. A prior study showed that patients with vaginal foreign bodies were between 1.5 and 14.8 years, with 3–10 years being the period of peak incidence (25), which is similar with our study. Hence, preschool girls require more careful care, more comfortable parenting pattern and necessary psychological referral and intervention.

The vaginal micro-flora is a complicated environment, composed of varying microbiological species in variable quantities and relative proportions. When this ecosystem gets disrupted, the vaginal epithelium is less protected, and vaginal infection sets in. Typically, vaginal infections are characterized by a shift in microbial communities that include a progressive replacement of certain *Lactobacillus species* by pathogenic or opportunistic microorganisms. This microbial shift can lead to different vaginal infections (26) The current study also explored common potential pathogens and the age distribution. The top five pathogens were *Haemophilus influenzae*, *Streptococcus pyogenes, Candida albicans, Escherichia coli and Staphylococcus aureus*. Earlier studies from various countries or regions showed *Streptococcus pyogenes* and *Haemophilus influenzae* were the two most identified pathogenic bacteria found in prepubertal girls with vulvovaginitis (11, 12, 27), which was consistent with our result. Previous study indicated AV can be frequently caused by *Group B streptococci*, *Escherichia coli*, *Staphylococcus aureus* (28). BV usually associated with several anaerobic or facultative bacteria, the most prevalent being: *G. vaginalis*; *Bacteroides* sp.etc. Another vaginal infection is VVC due to *Candida albicans*, *Candida glabrata*, and *Candida tropicalis*. The most prevalent form of vaginal infection in our study was belong to AV, because *Streptococcus pyogenes*, *Escherichia coli and Staphylococcus aureus* are three of the five common pathogens. AV is still known about its global epidemiology and implications, when comparing BV and VC, especially to young girls. Our result was in accordance with a previous study from Greece, which showed AV was more frequent in pubertal under-aged females, BV in reproductive age adult females (29). To adults, *Garderella vaginalis* appears to be the most virulent BV-associated anaerobe, which influences the biofilm formation (30). However, in the current study, *Garderella.* vaginalis has not been detected, which need further investigation. The VVC was mostly in 7–18 years old, especially in late adolescent girls (77.78%). The main pathogen was *C. albicans*. High estrogen levels and glycogen secretion in late adolescent girls changed the internal vaginal environment, such as pH values, and enhanced the adherence of fungi to cause VVC infection (28).In addition, the reason may also have been due to a stressful life in preparing for the College Entrance Examination, which may easily have caused VVC infection by impaired immunity. Moreover, VVC infection can be due to the growth and reproduction of fungi in a warm and humid environment, which also explains why VVC typically intensifies in July and August. Recurrence of VVC is also a problem for adolescent girls. In our study, follow-up times of girls aged 7–18 years old are more frequent than girls aged 0–6 years old. The high prevalence, substantial morbidity, and economic losses of recurrent vulvovaginal candidiasis exist not only in adults but also adolescents, which requires better solutions and improved quality of care for affected women (29).

The present study is the first retrospective study with a large sample size and comprehensive analysis exploring the spectrum of genital inflammation in pediatric and adolescent patients in China. The large sample size, long-time span, and wide range of disease conditions provide a better understanding of genital inflammation problems among Chinese girls. In addition, this study also analyzes common pathogenic data to supplement further data on the pathogens of pediatric female reproductive system diseases in China. However, the present study also has certain limitations. The basic demographic information obtained through electronic medical record data in this study was limited to pathogens detection and diagnosis, so the patients' daily health awareness, behaviors and clinical presentation could not be further explored, and only preliminary etiological hypotheses could be proposed. In addition, certain pathogens (e.g., *Garderella,* viruses) cannot be grown in existing culture media, which hindered us from fully understanding the pathogens of genital inflammation among girls. In future, we will improve the methods to reveal the accurate relationship between genital inflammation and vaginal dysbiosis. Moreover, it is a single-center study in which the population was mostly from within Zhejiang Province, and multicenter studies in China would be investigated in future. This would be more representative of the overall situation of genital condition among girls in China.

Conclusion

The patterns and epidemiological characteristics of genital inflammation at the first clinical visit in China have seldom been reported. Our study helps to fill this gap by examining the pattern of diagnoses among patients at the first clinical visit over an 8-year period in China. Overall, non-specific vulvovaginitis, lichen sclerosis, and labial adhesion were common issues for pediatric and adolescent patients with genital inflammation. The peak age of the first visit was 0–6 years old. The specific disease pattern varied among different age groups. Our study also reveals an increased number of genital inflammation patients in China over the decades, emphasizing the need for more public awareness, health education, healthcare services, and epidemiological research in this field.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by The study was approved by the Human Subjects

Committees of the Children's Hospital, Zhejiang University School of Medicine (approval number 2019-IBR-103). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

HG, MZ, and LS collected clinical data. HG and YZ did the conceptualization, design work, and wrote the original draft. YP, MZ, YQ, and SH contributed to the data analysis. SC, PS, KT, and CY supervised the research. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1073886/full#supplementary-material

References

1. Lanis A, Talib HJ. Prepubertal and adolescent Vulvovaginitis: what to do when a girl reports vaginal discharge. *Pediatr Ann*. (2020) 49:e170–5. doi: 10.3928/19382359-20200317-01

2. Sun L, Jiang Y. Patterns of pediatric and adolescent gynecologic problems in China: a hospital-based retrospective study of 97,252 patients. *J Pediatr Adolesc Gynecol*. (2022) 35:444–9. doi: 10.1016/j.jpag.2022.01.010

3. McGreal S, Wood PL. A study of paediatric and adolescent gynaecology services in a British district general hospital. *BJOG*. (2010) 117:1643–50. doi: 10.1111/j.1471-0528.2010.02724.x

4. Jiang L, Yan Y. Inflammation and endometriosis. *Front Biosci*. (2016) 21:941–8. doi: 10.2741/4431

5. van Schalkwyk J, Yudin MH. Vulvovaginitis: screening for and management of trichomoniasis, vulvovaginal candidiasis, and bacterial vaginosis. *J Obstet Gynaecol Can*. (2015) 37:266–74. doi: 10.1016/S1701-2163(15)30316-9

6. Kaida A, Dietrich JJ. A high burden of asymptomatic genital tract infections undermines the syndromic management approach among adolescents and young adults in South Africa: implications for HIV prevention efforts. *BMC Infect Dis*. (2018) 18:499. doi: 10.1186/s12879-018-3380-6

7. Tao X, Ge S-Q. Relationships between female infertility and female genital infections and pelvic inflammatory disease: a population-based nested controlled study. *Clinics*. (2018) 73:e364. doi: 10.6061/clinics/2018/e364

8. Loveless M, Myint O. Vulvovaginitis- presentation of more common problems in pediatric and adolescent gynecology. *Best Pract Res Clin Obstet Gynaecol*. (2018) 48:14–27. doi: 10.1016/j.bpobgyn.2017.08.014

9. Marcell AV, Burstein GR. Sexual and reproductive health Care Services in the Pediatric Setting. *Pediatrics*. (2017) 140:2–10. doi: 10.1542/peds.2017-2858

10. Francis SC, Holm HC. Results from a cross-sectional sexual and reproductive health study among school girls in Tanzania: high prevalence of bacterial vaginosis. *Sex Transm Infect*. (2019) 95:219–27. doi: 10.1136/sextrans-2018-053680

11. Hu B-F, Hua C-Z. Microbiological findings of symptomatic vulvovaginitis in chinese prepubertal girls. *J Pediatr Adolesc Gynecol*. (2021) 34:799–804. doi: 10.1016/j.jpag.2021.05.012

12. Bruins MJ, Dos Santos CO. Bacterial agents in vulvovaginitis and vaginal discharge: a 10-year retrospective study in the Netherlands. *Eur J Clin Microbiol Infect Dis*. (2021) 40:2123–8. doi: 10.1007/s10096-021-04265-8

13. Beyitler İ, Kavukcu S. Clinical presentation, diagnosis and treatment of vulvovaginitis in girls: a current approach and review of the literature. *World J Pediatr*. (2017) 13:101–5. doi: 10.1007/s12519-016-0078-y

14. Zhang Y, Liu T. STROBE-sequencing analysis of the vaginal microecology of 4- to 6-year-old girls in Southwest China. *Medicine (Baltimore)*. (2021) 100:e25362. doi: 10.1097/MD.0000000000025362

15. Sanfilippo JS, Lara-Torre E. *Sanfilippo's textbook of pediatric and adolescent gynecology*. Boca Raton: CRC Press (2019). 358 p.

16. Itriyeva K. Evaluation of vulvovaginitis in the adolescent patient. *Curr Probl Pediatr Adolesc Health Care*. (2020) 50:100836. doi: 10.1016/j.cppeds.2020.100836

17. Pitrelli N, Sturloni G. Infectious diseases and governance of global risks through public communication and participation. *Ann Ist Super Sanita*. (2007) 43:336–43.

18. Shannon CL, Klausner JD. The growing epidemic of sexually transmitted infections in adolescents: a neglected population. *Curr Opin Pediatr*. (2018) 30:137–43. doi: 10.1097/MOP.0000000000000578

19. Chen X, Chen L. *Haemophilus influenzae* vulvovaginitis associated with rhinitis caused by the same clone in a prepubertal girl. *J Obstet Gynaecol Res*. (2017) 43:1080–3. doi: 10.1111/jog.13311

20. Qi Y, Shi P. Characteristics of childhood allergic diseases in outpatient and emergency departments in Shanghai, China, 2016-2018: a multicenter, retrospective study. *BMC Pediatr*. (2021) 21:409. doi: 10.1186/s12887-021-02880-0

21. Singh N, Ghatage P. Etiology, clinical features, and diagnosis of vulvar lichen Sclerosus: a scoping review. *Obstet Gynecol Int*. (2020) 2020:7480754–8. doi: 10.1155/2020/7480754

22. Tammaro A, Magri F. Lichen sclerosus et atrophicus and allergic contact dermatitis: a significant association. *J Cosmet Dermatol*. (2019) 18:1935–7. doi: 10.1111/jocd.12915

23. Ding X, Wang J. Individual, prenatal, perinatal, and family factors for anxiety symptoms among preschool children. *Front Psychol*. (2021) 12:778291. doi: 10.3389/fpsyt.2021.778291

24. Ma W, Sun Y-F. Vaginal foreign bodies in children: a single-center retrospective 10-year analysis. *Pediatr Surg Int*. (2022) 38:637–41. doi: 10.1007/s00383-022-05075-5

25. Kim H, Chai SM. Clinical and microbiologic characteristics of vulvovaginitis in Korean prepubertal girls, 2009-2014: a single center experience. *Obstet Gynecol Sci*. (2016) 59:130–6. doi: 10.5468/ogs.2016.59.2.130

26. Salinas AM, Osorio VG. Vaginal microbiota evaluation and prevalence of key pathogens in ecuadorian women: an epidemiologic analysis. *Sci Rep*. (2020) 10:18358. doi: 10.1038/s41598-020-74655-z

27. Xu L, Hu Z. Analysis of characteristics of vulvo-vaginal infections in 14- to 18-year-old girls in late puberty. *J Int Med Res*. (2020) 48:300060520946506. doi: 10.1177/0300060520946506

28. Donders GGG, Vereecken A. Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis. *BJOG*. (2002) 109:34–43. doi: 10.1111/j.1471-0528.2002.00432.x

29. Sianou A, Galyfos G. Prevalence of vaginitis in different age groups among females in Greece. *J Obst Gynaecol*. (2017) 37:790–4. doi: 10.1080/01443615.2017.1308322

30. Machado A, Cerca N. Influence of biofilm formation by Gardnerella vaginalis and other anaerobes on bacterial vaginosis. *J Infect Dis*. (2015) 212:1856–61. doi: 10.1093/infdis/jiv338

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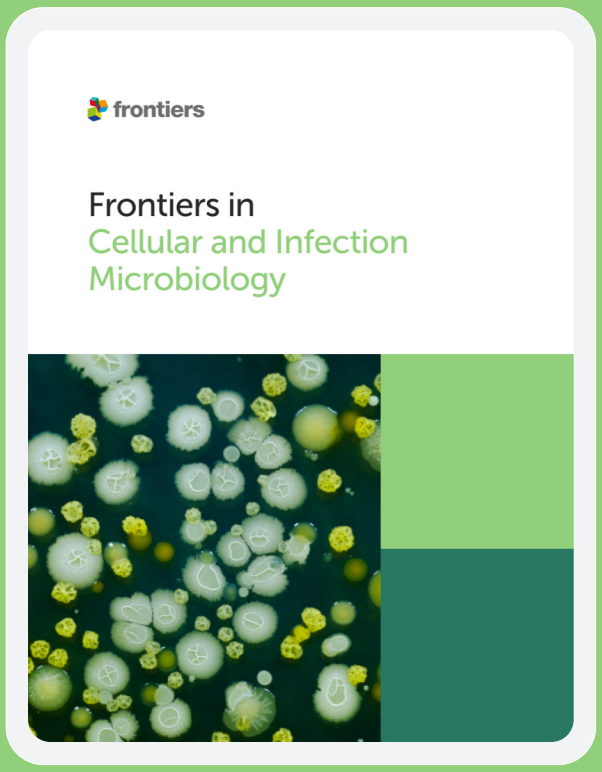

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