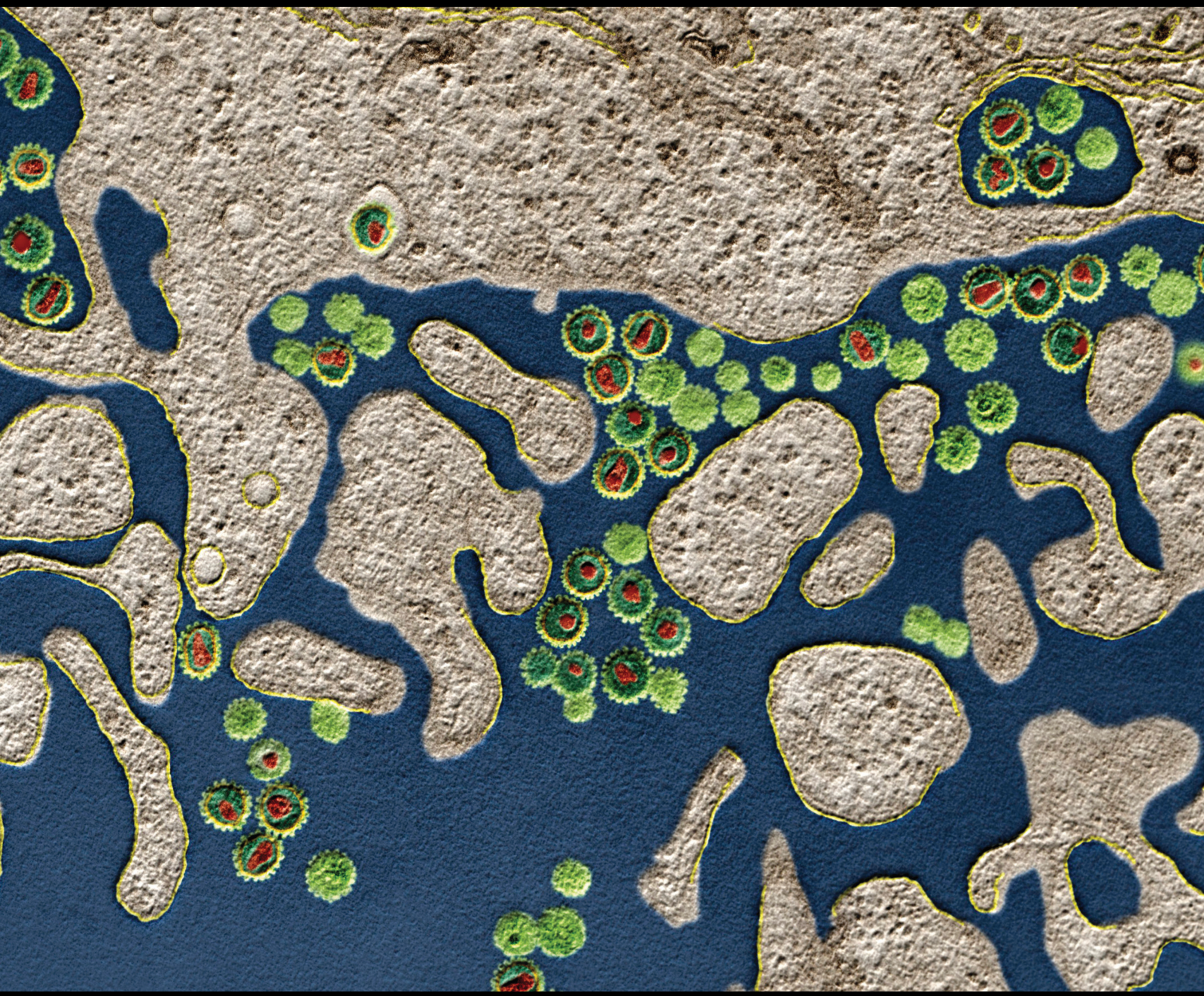
Journal of Immunology Research

Immunological Mechanisms in Vaginal Microbiota

Lead Guest Editor: Ana Katherine Gonçalves
Guest Editors: Paulo Cesar Giraldo, Pedro Vieira-Baptista, and José Eleutério Júnor



Immunological Mechanisms in Vaginal Microbiota

Journal of Immunology Research

Immunological Mechanisms in Vaginal Microbiota

Lead Guest Editor: Ana Katherine Gonçalves
Guest Editors: Paulo Cesar Giraldo, Pedro Vieira-Baptista, and José Eleutério Júnor

Contents

Hindawi
Journal of Immunology Research
Volume 2022, Article ID 3525735, 8 pages
https://doi.org/10.1155/2022/3525735

Research Article

Vaginal Microbial Environment Skews Macrophage Polarization and Contributes to Cervical Cancer Development

Guannan Zhou,[1,2] **Fangyue Zhou**,[3] **Yuanyuan Gu**,[1,2] **Menglei Zhang**,[1] **Ganrong Zhang**,[1] **Fang Shen**,[1] **Keqin Hua**,[1,2] **and Jingxin Ding**[1,2]

[1]*Department of Gynecology, The Obstetrics and Gynecology Hospital of Fudan University, 419 Fang-Xie Road, Shanghai 200011, China*
[2]*Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China*
[3]*The International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China*

Correspondence should be addressed to Keqin Hua; huakeqinjiaoshou@163.com and Jingxin Ding; djxdd@sina.com

Received 23 May 2022; Revised 5 July 2022; Accepted 14 July 2022; Published 9 August 2022

Academic Editor: José Eleutério

As a common female reproductive system malignancy, cervical cancer (CC) disturbs numerous women's health. This study demonstrates the role of the vaginal microbial environment (*Peptostreptococcus anaerobius*) in cervical cancer. Functional assays, including cell proliferation assay, tube formation assay, and immunofluorescence staining, revealed the effect of *Peptostreptococcus anaerobius*-treated macrophages on cell proliferation and the angiogenesis process. The tube formation assay disclosed the function of *Peptostreptococcus anaerobius*-treated macrophages on angiogenesis. In vivo assays were also established to explore the impact of *Peptostreptococcus anaerobius*-treated macrophages on tumor migration. The results revealed that *Peptostreptococcus anaerobius*-induced macrophages boosted cervical cancer migration and angiogenesis both in vitro and in vivo. Then, this study unveiled that *Peptostreptococcus anaerobius*-induced macrophage secreted VEGF to stimulate the angiogenesis in cervical cancer. As a whole, *Peptostreptococcus anaerobius*-induced macrophage facilitates cervical cancer development through modulation of VEGF expression.

1. Introduction

It was widely acknowledged that the healthy physiological process depends on the healthy and balanced vaginal microbial ecosystem [1]. As the development of sequencing technology, the role of the microbial environment in the body is widely demonstrated [2, 3]. Many studies have reported that bacterial vaginosis is associated with many reproductive issues [4]. For example, the adverse obstetric outcomes are related to pelvic inflammatory diseases [5] and PCOS [4, 6]. Several studies [7–9] have reported that the variation of the vaginal microbe composition influences the infection status of *Human papillomavirus* (HPV). Our previous study indicated that the composition changes are correlated with the cervical precancer lesions and HPV infections [10]. However, whether the vaginal microbes determine the immune response in the tumor microenvironment and further affect the tumor progression including migration and angiogenesis is unclear.

Cervical cancer is one of the main malignancies in the female reproductive system [11], while the persistent high-risk HPV infection [12] is the main cause of most cervical precancer and cervical cancers. The increasing evidence indicates that the vaginal microbial environment is associated with the progression of female diseases, including infertility, PCOS, inflammation [13], and cancers [14, 15]. So, what is the relationship between vaginal microbe and cervical cancer? And how the vaginal microbial environment

exerts the functions in the progression of cervical cancer is still unclear.

In this study, we figured out the differential expressed vaginal microbes in cervical cancer women and then demonstrated the effect of the *Peptostreptococcus anaerobius* on the macrophage polarization, further regulating the angiogenesis in vitro and migration in vivo via secreting VEGF.

2. Methods and Materials

2.1. Cell Culture. Cervical cancer SiHa cells and SiHa-Luciferase were gifts from the laboratory of the Obstetrics and Gynecology Hospital of Fudan University and maintained in RPMI-1640 medium (E600028, Sangon Biotech, Songjiang, Shanghai, China), supplemented with 10% FBS (Gibco, Guangzhou, Guangdong, China), 1% penicillin (Sangon Biotech, Songjiang, Shanghai, China), and 1% streptomycin in a humidified incubator with 5% CO_2 at 37°C. HUVEC cells were maintained in DMEM medium supplemented with 10% FBS as well as 1% penicillin & streptomycin in a humidified incubator with 5% CO_2 at 37°C.

2.2. Ethics Approval. The study was approved by the institutional review board of the Obstetrics and Gynecology Hospital of Fudan University. All the study processes were implemented based on the Declaration of Helsinki. Also, the study obtained oral informed consents and written informed consents. All animal experiments were housed and maintained in a standard environment, and the protocol was reviewed and approved by the animal care and research committee of Fudan University.

2.3. Tissue Samples. The ethical approval of this study was acquired from the ethics committee of the hospital, and written informed consents were provided by all participants. A total of 20 of cervical squamous cell carcinoma samples (10 cervical squamous cell carcinoma samples with lymphatic metastasis positive and 10 cervical squamous cell carcinoma samples with lymphatic metastasis negative as control) were acquired from patients who receive surgery and were collected for this study between January 2019 and March 2020. The exclusion criteria were as follows: (i) history of other drug treatment before surgery, (ii) history of cervical conization surgery, (iii) other pathological types of cervical cancer carcinoma, (iv) other female reproductive system malignancies, and (v) non-postmenopausal. Tissue samples were all snap frozen in liquid nitrogen and preserved at −80°C, for further measurement.

2.4. Cell Viability Assay. The cell viability of HUVEC cells was evaluated by CCK8 assays. Totally, 10^4 cells/well were seeded into the 96-well plate with 100 μL DMEM for 12 h and cells were added with *Peptostreptococcus anaerobius*-treated macrophage medium, blank bacterium medium-treated macrophage medium, and PBS for 48 h. Then, the CCK-8 kit was added into the medium in wells for 1 hour at 37°C. The cell viability was evaluated by the OD450 value of each well.

2.5. Tube-Formation Assay. Matrigel was evenly distributed to every well in a 96-well plate for 30 min at 37°C. HUVEC cells at early passage were prepared and added into each well for 3 hours and captured under the microscope. Tube formation was assessed under the microscope.

2.6. ELISA Assay. After being treated with diverse medium, the cell supernatant sample collection was conducted. Briefly, the detection of the serum levels of VEGF was conducted by the ELISA kits (Yanhui, Shanghai, China) ($^{***}p < 0.001$).

2.7. Immunofluorescence Assay. HUVEC cells were seeded on a slide in the 24-well plate. Immunofluorescence images of VEGFR2 expression on HUVECs were captured, after treatment with indicated media (Peptostreptococcus anaerobius treated macrophage media) stimulation for 72h. Cells were fixed with 10% paraformaldehyde, the VEGFR were stained by FITC-VEGFR antibody, and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Millipore) for 10 min at 4°C. Images were taken using a Leica fluorescent microscope and a TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, GER).

2.8. Animal Assay. Mice were divided into three groups: (i) *Peptostreptococcus anaerobius*-treated macrophage group, (ii) blank medium-treated macrophage group, and (iii) PBS group. Female athymic nude mice (4 weeks old) were purchased from JieSiJie Laboratory Animal Co. Ltd., Shanghai, China. For bioluminescence evaluation in the mouse model, we cultured SiHa-Luciferase stable transfected cells. SiHa-Luciferase cells (2×10^6/mL) were seeded into nude mice via intraperitoneal injection. Medium gathered from *Peptostreptococcus anaerobius*-treated macrophages, medium from blank medium-treated macrophages, and PBS were injected into mice intraperitoneally for two times a week and totally 3 weeks. Mice were analyzed by a live imaging upon animals.

2.9. Data Analysis. In this study, we conducted the correlation analysis not only for the gene expression evaluation but also for the survival prognosis evaluation in cervical carcinoma, which is based on the TCGA database (https://tcga-data.nci.nih.gov). Furthermore, we conducted analysis (including the expression analysis and the survival analysis) based on the GEPIA2 website (http://gepia2.cancer-pku.cn/). All the experiments were repeated in triplicate, and experimental results were expressed as the means ± standard deviation (S.D.). We use Student's t-test or one-way ANOVA to determine statistical probabilities, with a p value below 0.05 as a significant level. And we used the SPSS 25.0 software (IBM Corp., Armonk, NY) to analyze the data. Gene linear correlation was analyzed by Pearson correlation analysis.

3. Results

3.1. Peptostreptococcus anaerobius Is Upregulated Expressed in Cervical Cancer Cervicovaginal Lavage Fluid and Tissues. At first, the expression of *Peptostreptococcus anaerobius* in 20 participants (including 10 women (cervical squamous cell carcinoma lymphatic metastasis positive) with

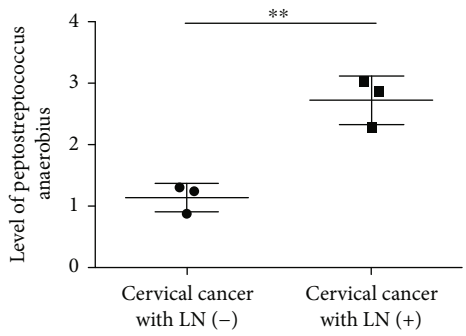


Figure 1: The level of *Peptostreptococcus anaerobius* in cervical cancer.

cervicovaginal lavage fluid and 10 women (cervical squamous cell carcinoma lymphatic metastasis negative, as control) with cervicovaginal lavage fluid) was examined. As depicted in Figure 1, it was revealed that *Peptostreptococcus anaerobius* was significantly highly expressed in cervicovaginal lavage fluid of cervical cancer women, when compared with healthy women. As depicted in our previous study, the level of *Peptostreptococcus anaerobius* in cervical cancer lesion was higher than that of nontumor women.

3.2. The Relationship between the M2 Phenotype and Peptostreptococcus anaerobius in Cervical Cancer. To investigate the correlation between infiltration of tumor-associated macrophages and *Peptostreptococcus anaerobius* in cervical cancer, we performed IHC staining to detect the M2 macrophage marker CD206 in cervical cancer tissues derived from women with *Peptostreptococcus anaerobius* and cervical cancer tissues derived from women without *Peptostreptococcus anaerobius*. As shown in Figure 2(a), we observed elevated infiltration of CD206-positive M2 macrophage infiltration in cervical cancer tissues from women with *Peptostreptococcus anaerobius*, when compared with women without *Peptostreptococcus anaerobius*. In addition, overall survival analysis showed that the overall survival rate of the higher expression of the CD206-positive group in cervical cancer was better than that of lower expression of the CD206-positive group. Also, we conducted the analysis about the survival prognosis in cervical cancer patients. As depicted in Figure 2(b), the increased expression level of CD206 was relevant to the lower survival rate and inferior prognosis, while the decreased expression level of CD206 was relevant to the higher survival rate and superior prognosis.

3.3. The Effect of Peptostreptococcus anaerobius to Induce the Macrophage into the M2 Phenotype. To determine whether *Peptostreptococcus anaerobius* induced M2 polarization of macrophages, we first selected THP-1 cells and induced THP-1 cells into the M0 macrophage by PMA and then treated M0 macrophages with collected *Peptostreptococcus anaerobius* medium. As depicted in Figure 3, the results showed that the expression of M2 markers (CD206) in PMA-treated THP-1 cell-administered blank medium or *Peptostreptococcus anaerobius* medium was apparently lower or higher, respectively. Taken together, the abovementioned results confirm that *Peptostreptococcus anaerobius* can induce M2 polarization of macrophages.

3.4. The Effect of Peptostreptococcus anaerobius-Treated Macrophage to Induce the Angiogenesis. In order to analyze the effect of *Peptostreptococcus anaerobius*-treated macrophage on regulating the angiogenesis process, we conducted the viability assay and tube formation assay by using the HUVEC cells. As shown in Figure 4, the tube formation assay revealed that the induction of *Peptostreptococcus anaerobius*-treated macrophages on tube formation efficiency was higher than the blank medium-treated macrophages and PBS-treated macrophages.

3.5. The Peptostreptococcus anaerobius-Treated Macrophages Secrete VEGF to Induce Angiogenesis. Since *Peptostreptococcus anaerobius*-treated macrophages expressed markers of the M2 phenotype, and M2 macrophages were reported to secret cytokines and growth factors, such as VEGF and PDGF. In order to explore how the *Peptostreptococcus anaerobius*-treated macrophages upregulate the ability of angiogenesis, we conducted the ELISA assay to evaluate the VEGF expression of the *Peptostreptococcus anaerobius*-treated macrophages, blank bacteria medium-treated macrophage medium, and control groups. As depicted in Figure 5(a), the results revealed that VEGF expression was significantly upregulated in *Peptostreptococcus anaerobius*-treated macrophage medium than in the blank bacterium-treated macrophage medium or the control group. While we added the VEGF receptor protein into the HUVECs with medium from *Peptostreptococcus anaerobius*-treated macrophages, the induced angiogenesis process was inhibited. What is more, in Figure 5(b), the *Peptostreptococcus anaerobius*-treated macrophage medium could increase the expression level of VEGFR2 in HUVEC cells. In addition, as depicted in Figures 5(c) and 5(d), the cervical cancer tissue expressed a higher level of VEGF and the increased expression level of VEGF was relevant to the lower survival rate and inferior prognosis.

3.6. The Effect of Peptostreptococcus anaerobius-Treated Macrophages on Inducing the Migration of Cervical Cancer in the Animal Model. In in vivo assay, we used the SiHa-luciferase cancer cells for evaluating the effect of *Peptostreptococcus anaerobius*-treated macrophages on the cervical cancer. We divided animals into three groups: *Peptostreptococcus anaerobius*-treated macrophage group, blank medium-treated macrophage group, and PBS-treated group. Luminescence of cervical cancer cells was evaluated by an in vivo imaging system to demonstrate the cancer metastasis in vivo. As demonstrated in Figure 6(a), *Peptostreptococcus anaerobius*-treated macrophages enhanced the migration of cervical cancer in vivo, when compared with blank medium-treated macrophages or PBS. In addition, the VEGFR2 expression level in tumor resected from the *Peptostreptococcus anaerobius*-treated macrophage group is higher than that from the blank medium-treated macrophage groups or PBS group (Figure 6(b)).

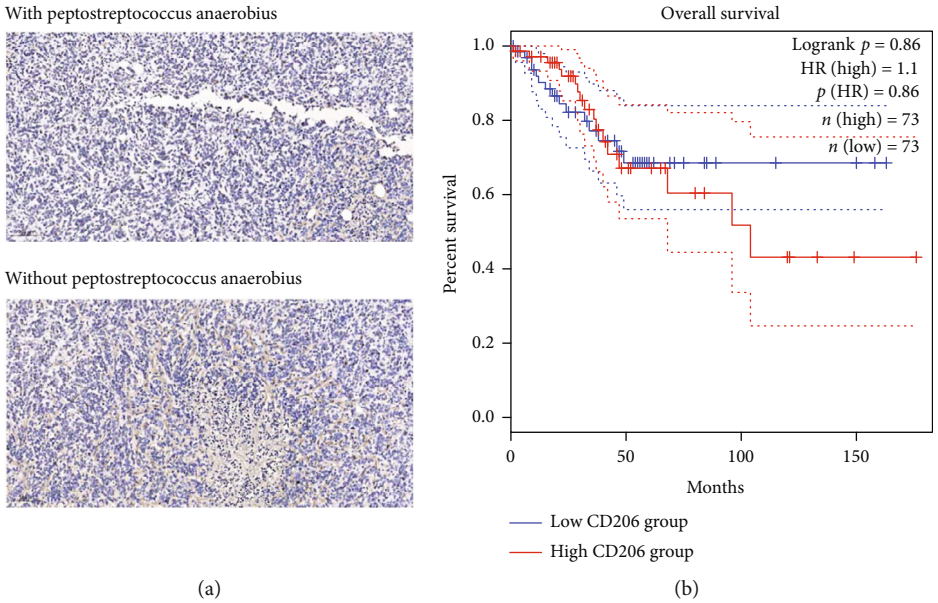


Figure 2: The expression of CD206 in cervical cancer. (a) The IHC staining of cervical carcinoma tissues upon the CD206 in cervical cancer tissues from women with *Peptostreptococcus anaerobius* (down) and in cervical cancer tissues from women without *Peptostreptococcus anaerobius* (upper). (b) The analysis of CD206 expression via TCGA set base: the increased expression level of CD206 was relevant to the lower survival rate and inferior prognosis (blue curves); the decreased expression level of CD206 was relevant to the higher survival rate and superior prognosis (red curves).

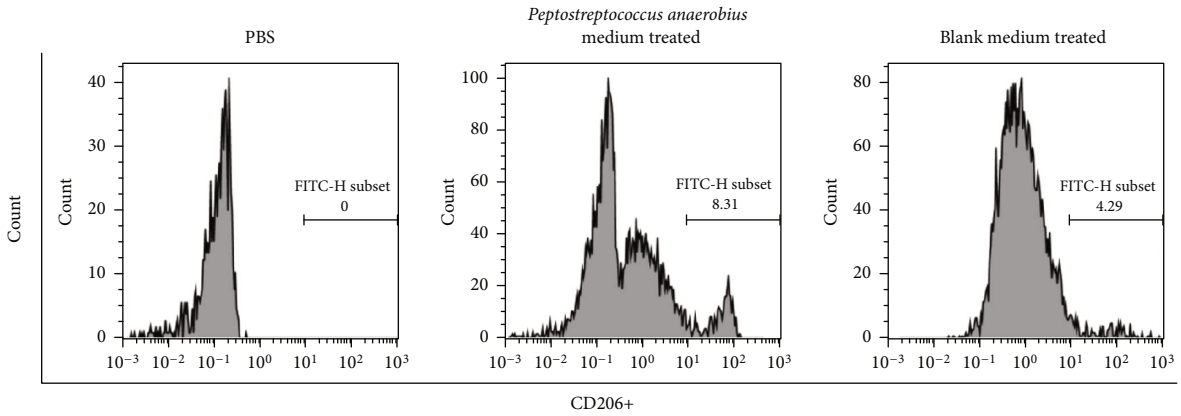


Figure 3: The effect of *Peptostreptococcus anaerobius* upon the polarization of macrophages: the expression of CD206 (M2 macrophage markers) in THP-1 cells when treated with PBS or *Peptostreptococcus anaerobius* medium or blank medium.

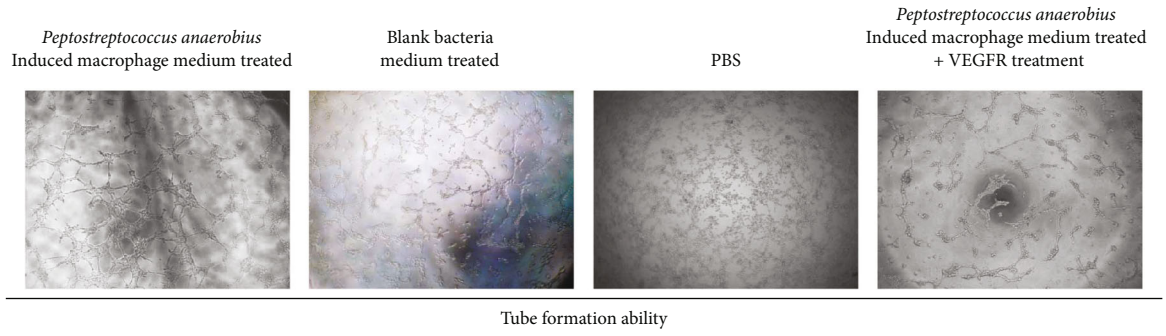


Figure 4: The effect of *Peptostreptococcus anaerobius* upon tube formation for detecting angiogenesis ability. The tube formation ability of HUVECs after being treated with medium from *Peptostreptococcus anaerobius*-induced macrophages, blank bacteria medium-induced macrophages, PBS, and medium from *Peptostreptococcus anaerobius*-induced macrophages plus VEGFR.

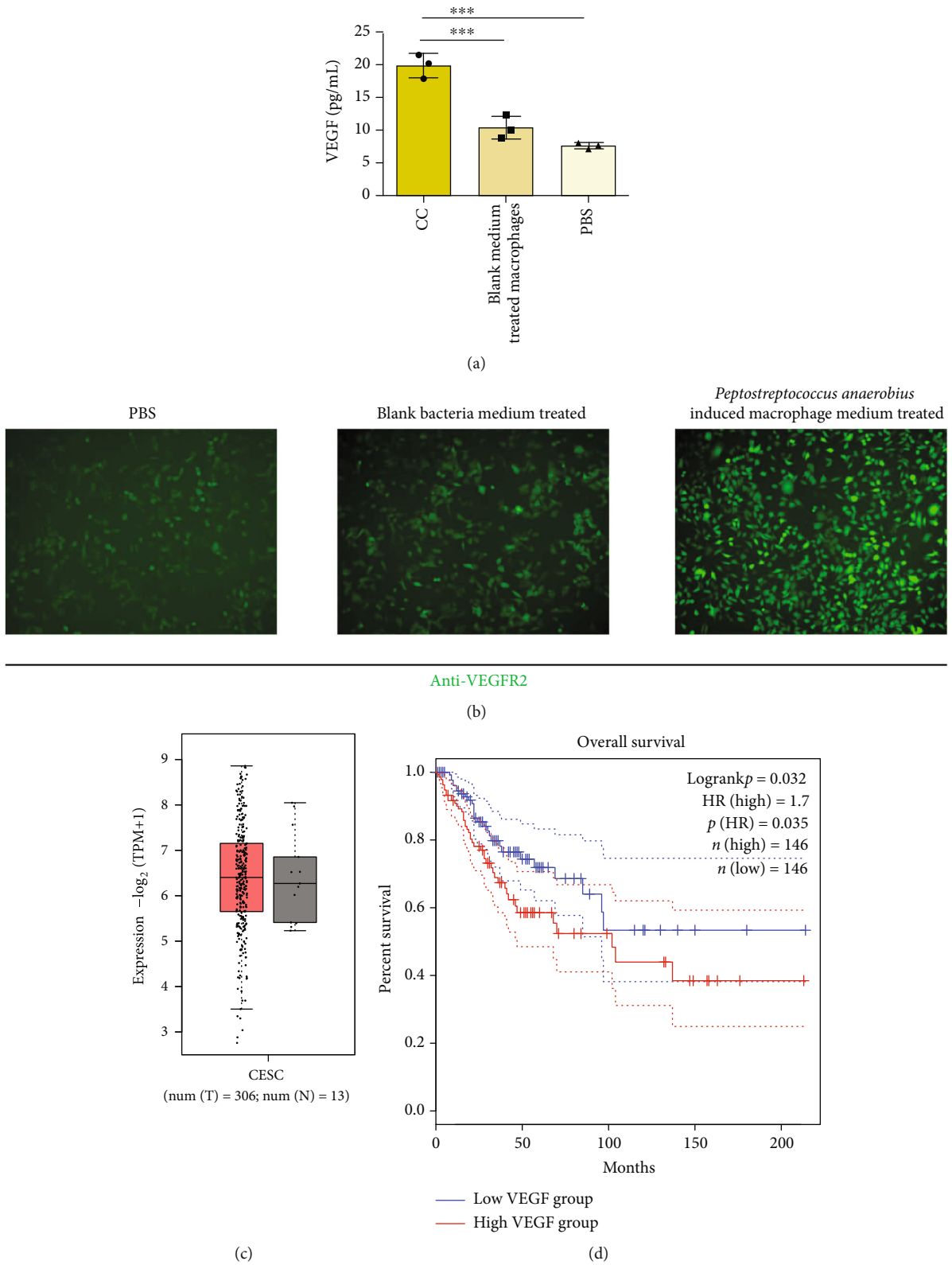


Figure 5: The expression level of VEGF from macrophages treated with *Peptostreptococcus anaerobius*. (a) The expression level of VEGF from macrophage medium after being treated with medium from *Peptostreptococcus anaerobius*-induced macrophages, blank bacteria medium-induced macrophages, and PBS. (b) The VEGFR expression on HUVECs after being treated with medium from *Peptostreptococcus anaerobius*-induced macrophages, blank bacteria medium-induced macrophages, and PBS. The expression analysis (c) and overall survival analysis (d) of VEGF in cervical cancer via TCGA set base.

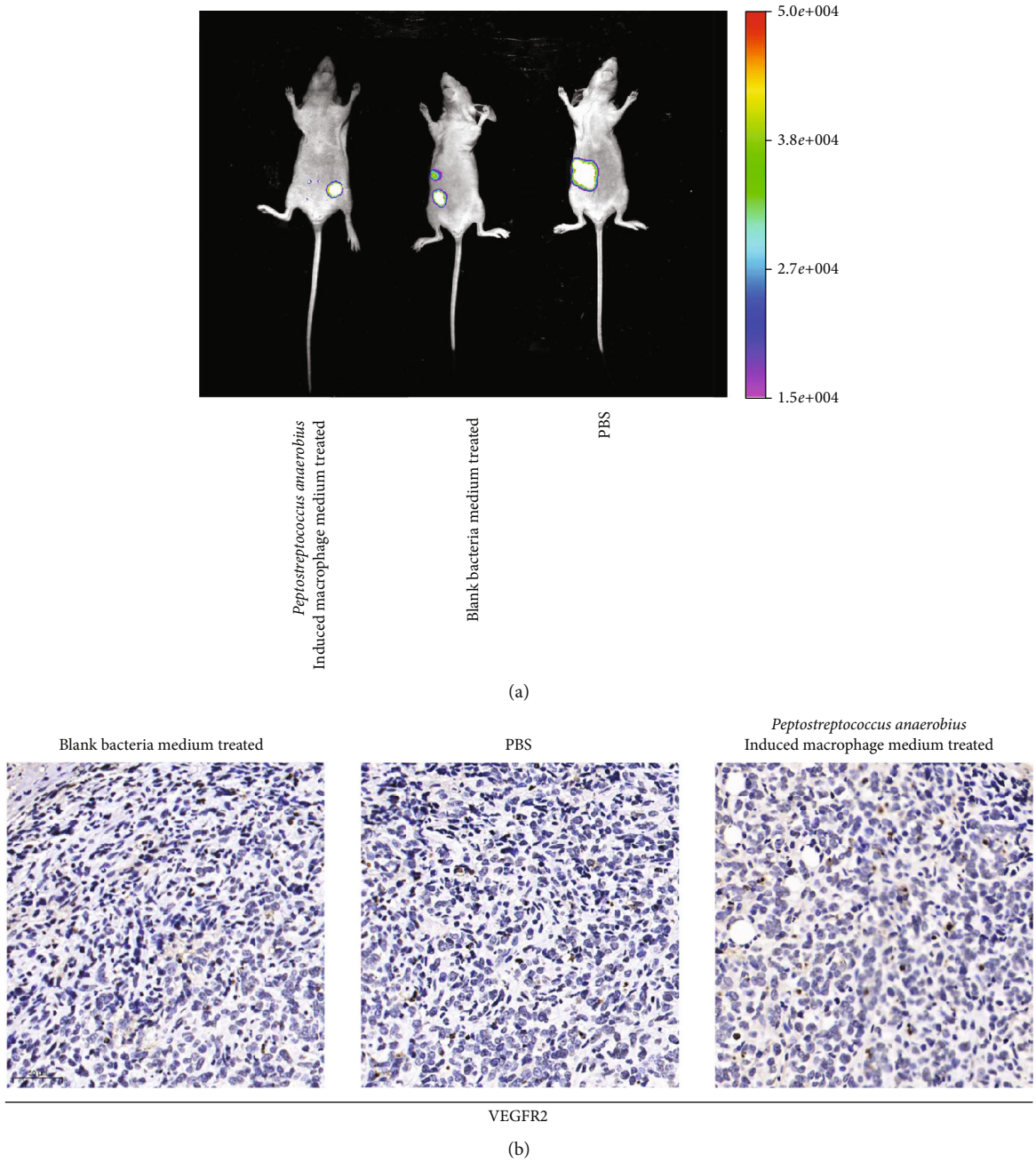


Figure 6: The effect of VEGF from macrophages treated with *Peptostreptococcus anaerobius* on the migration in cervical cancer in vivo. (a) Bioluminescent images were visualized to evaluate the metastasis of cervical cancer cells after being treated with *Peptostreptococcus anaerobius*-induced macrophage-conditioned medium, blank bacteria medium-induced macrophage-conditioned medium, and PBS in the mouse model. Luminescence of cervical cancer cells was evaluated by an in vivo imaging system, in which the size of the luminescence area represents the metastasis ability. (b) The IHC staining for detecting the VEGFR expression in tumor after being treated with blank bacteria medium-induced macrophage-conditioned medium, PBS, and *Peptostreptococcus anaerobius*-induced macrophage-conditioned medium.

4. Discussion

Cervical cancer is the one of the main female reproductive system malignancies, which disturbs the health of women around the world. Although, with the development of screening strategy [16] and the popularization of HPV vaccine [17], the morbidity and mortality decreased in developed countries [18]. However, cervical carcinomas still result in numerous deaths in developing countries. Nowadays, increasing evidence indicate that vaginal microbes are associated to the disease process [19], including inflammations [20], infertility [21], and cancers [22]. In the current study, we demonstrated that *Peptostreptococcus anaerobius*, a kind of vaginal microbe, is expressed rarely in healthy women, promotes the macrophage polarization in the tumor microenvironment, and further induces the angiogenesis process

in vitro and migration in vivo. Mechanistically, the *Peptostreptococcus anaerobius*-induced macrophage expressed the M2 phenotype and the *Peptostreptococcus anaerobius*-induced macrophage could secret VEGF to induce the angiogenesis process. VEGF is a specific angiogenesis factor and could stimulate endothelial tube formation to generate new vessels. A previous study [23] revealed that VEGF could induce the angiogenic process upon endothelial cells. These findings indicate that the vaginal microbes exert certain functions in the development of cervical cancer.

It was of significance to demonstrate the effect of the vaginal microbial environment in the physiological process and pathological process in the female reproductive system. It was reported [24, 25] that there are significant differences in vaginal microbiome between cancer microenvironment and noncancer microenvironment women, but less studies reported the mechanisms among these associations. In this study, we explore the effect of *Peptostreptococcus anaerobius* on macrophage polarization and further angiogenesis and also explore the certain mechanisms of these effects.

To our knowledge, this is the first study to demonstrate the effect of *Peptostreptococcus anaerobius* on macrophage polarization in the tumor microenvironment in cervical cancer. This study is based on our previous analysis by means of comparing the expression differences among the different status of cervical precancer lesions.

There are also many limitations to this current study. Firstly, more relative samples of cervical cancer tissues are needed to verify the relations between the macrophage infiltration and vaginal microbe; also, more samples of cervicovaginal lavage fluid are needed in the future study. Secondly, further more studies shoube be conducted to figure out if there are other vaginal microbials could play roles in regulating the vaginal microenvironment in cervical cancer. What is more, further experiments should explore whether the expression of specific vaginal microbes indicates the specific prognosis in cervical cancer.

5. Conclusions

We demonstrated that vaginal microbial *Peptostreptococcus anaerobius* could contribute to cervical cancer progression via inducing M2 macrophage polarization. In addition, we revealed that *Peptostreptococcus anaerobius*-induced macrophage could promote the angiogenesis in vitro and in vivo. Furthermore, we found that *Peptostreptococcus anaerobius*-induced macrophage could promote the angiogenesis via secreting VEGF. Thus, our study elucidated a novel molecular mechanism promoting cervical cancer underlying the interaction between vaginal microbes, macrophages, and tumor cells. These results will contribute to the new insights of the development of cervical cancer. Also, the results would contribute to the effective preventive and therapeutic strategies for cervical cancer. More importantly, high *Peptostreptococcus anaerobius* in cervicovaginal lavage fluid was correlated with cervical cancer, suggesting that it may be a promising biomarker for liquid biopsy and predicting the risk of cervical cancer in the future. In addition, targeting the vaginal microbial-mediated crosstalk between tumor cells and macrophages may provide novel strategies for the treatment of cervical cancer.

Data Availability

Data are available form the corresponding authors once needed.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Guannan Zhou, Fangyue Zhou, and Yuanyuan Gu contribute equally to this work; Keqin Hua and Jingxin Ding contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 91440107) and Natural Science Foundation of Shanghai (no. 21ZR1410400).

References

[1] J. Norenhag, J. A.-O. Du, M. A.-O. X. Olovsson, H. A.-O. Verstraelen, L. A.-O. Engstrand, and N. A.-O. X. Brusselaers, "The vaginal microbiota, human papillomavirus and cervical dysplasia: a systematic review and network meta-analysis," *BJOG : An International Journal of Obstetrics and Gynaecology*, vol. 127, no. 2, pp. 171–180, 2020.

[2] J. C. Borgogna, M. D. Shardell, E. K. Santori et al., "The vaginal metabolome and microbiota of cervical HPV-positive and HPV-negative women: a cross-sectional analysis," *BJOG : An International Journal of Obstetrics and Gynaecology*, vol. 127, no. 2, pp. 182–192, 2020.

[3] M. C. Eastment, J. E. Balkus, B. A. Richardson et al., "Association between vaginal bacterial microbiota and vaginal yeast colonization," *The Journal of Infectious Diseases*, vol. 223, no. 5, pp. 914–923, 2021.

[4] Y. Gu, G. Zhou, F. Zhou et al., "Gut and vaginal microbiomes in PCOS: implications for women's health," *Frontiers in Endocrinology*, vol. 13, no. 13, p. 808508, 2022.

[5] J. Ravel, I. Moreno, and C. Simón, "Bacterial vaginosis and its association with infertility, endometritis, and pelvic inflammatory disease," *American Journal of Obstetrics and Gynecology*, vol. 224, no. 3, pp. 251–257, 2021.

[6] X. Qi, C. Yun, L. Sun et al., "Gut microbiota-bile acid-interleukin-22 axis orchestrates polycystic ovary syndrome," *Nature Medicine*, vol. 25, no. 8, pp. 1225–1233, 2019.

[7] Y. Li, T. Yu, H. Yan et al., "Vaginal microbiota and HPV infection: novel mechanistic insights and therapeutic strategies," *Infection and Drug Resistance*, vol. 13, pp. 1213–1220, 2020.

[8] M. Usyk, C. A.-O. Zolnik, P. A.-O. Castle et al., "Cervicovaginal microbiome and natural history of HPV in a longitudinal study," *PLoS Pathogens*, vol. 16, no. 3, article e1008376, 2020.

[9] N. Brusselaers, S. Shrestha, J. van de Wijgert, and H. Verstraelen, "Vaginal dysbiosis and the risk of human papillomavirus and cervical cancer: systematic review and meta-

analysis," *American Journal of Obstetrics and Gynecology*, vol. 221, no. 1, pp. 9–18.e8, 2019.

[10] F. Y. Zhou, Q. Zhou, Z. Y. Zhu, K. Q. Hua, L. M. Chen, and J. X. Ding, "Types and viral load of human papillomavirus, and vaginal microbiota in vaginal intraepithelial neoplasia: a cross-sectional study," *Annals of Translational Medicine*, vol. 8, no. 21, p. 1408, 2020.

[11] A. Buskwofie, G. David-West, and C. A. Clare, "A review of cervical cancer: incidence and disparities," *Journal of the National Medical Association*, vol. 112, no. 2, pp. 229–232, 2020.

[12] E. M. Burd, "Human papillomavirus and cervical cancer," *Clinical Microbiology Reviews*, vol. 16, no. 1, pp. 1–17, 2003.

[13] L. M. Kindinger, D. A. MacIntyre, Y. S. Lee et al., "Relationship between vaginal microbial dysbiosis, inflammation, and pregnancy outcomes in cervical cerclage," *Science Translational Medicine*, vol. 8, no. 350, 2016.

[14] Y. Zhang, S. Qiu, Y. Guo, J. Zhang, X. Wu, and G. A.-O. Hong, "Diagnostic value of vaginal microecology, serum miR-18a, and PD-L1 for identifying HPV-positive cervical cancer," *Technology in Cancer Research & Treatment*, vol. 20, no. 20, 2021.

[15] E. Kudela, A. Liskova, M. Samec et al., "The interplay between the vaginal microbiome and innate immunity in the focus of predictive, preventive, and personalized medical approach to combat HPV-induced cervical cancer," *The EPMA Journal*, vol. 12, no. 2, pp. 199–220, 2021.

[16] Y. Y. Gu, G. N. Zhou, Q. Wang, J. X. Ding, and K. Q. Hua, "Evaluation of a methylation classifier for predicting precancer lesion among women with abnormal results between HPV16/18 and cytology," *Clinical Epigenetics*, vol. 12, no. 1, p. 57, 2020.

[17] K. Rosalik, C. Tarney, and J. A.-O. X. Han, "Human papilloma virus vaccination," *Viruses*, vol. 13, no. 6, p. 1091, 2021.

[18] K. T. Simms, J. Steinberg, M. Caruana et al., "Impact of scaled up human papillomavirus vaccination and cervical screening and the potential for global elimination of cervical cancer in 181 countries, 2020-99: a modelling study," *The Lancet Oncology*, vol. 20, no. 3, pp. 394–407, 2019.

[19] A. A.-O. Lev-Sagie, D. Goldman-Wohl, Y. Cohen et al., "Vaginal microbiome transplantation in women with intractable bacterial vaginosis," *Nature Medicine*, vol. 25, no. 10, pp. 1500–1504, 2019.

[20] A. A.-O. Lev-Sagie, D. Goldman-Wohl, Y. Cohen et al., "Vaginal microbiome transplantation in women with intractable bacterial vaginosis," *Frontiers in Cellular and Infection Microbiology*, vol. 11, no. 10, 2020.

[21] J. A.-O. Fettweis, M. G. Serrano, J. P. Brooks et al., "The vaginal microbiome and preterm birth," *Nature Medicine*, vol. 25, no. 6, pp. 1012–1021, 2019.

[22] M. Champer, A. M. Wong, J. Champer et al., "The role of the vaginal microbiome in gynaecological cancer," *BJOG : An International Journal of Obstetrics and Gynaecology*, vol. 125, no. 3, pp. 309–315, 2018.

[23] R. J. Epstein, "VEGF signaling inhibitors: more pro-apoptotic than anti-angiogenic," *Cancer Metastasis Reviews*, vol. 26, no. 3-4, pp. 443–452, 2007.

[24] M. Kyrgiou, A. Mitra, and A. B. Moscicki, "Does the vaginal microbiota play a role in the development of cervical cancer?," *Translational Research*, vol. 179, pp. 168–182, 2017.

[25] L. A.-O. Pagan, R. A. M. Ederveen, B. W. Huisman et al., "The human vulvar microbiome: a systematic review," *Microorganisms*, vol. 9, no. 12, p. 2568, 2021.

Hindawi
Journal of Immunology Research
Volume 2022, Article ID 1372926, 11 pages
https://doi.org/10.1155/2022/1372926

Research Article

Age-Stratified Analysis of Vaginal Microbiota Dysbiosis and the Relationship with HPV Viral Load in HPV-Positive Women

Mingzhu Li, Chao Zhao, Yun Zhao, Jingran Li, and Lihui Wei

Department of Obstetrics and Gynecology, Peking University People's Hospital, No. 11 Xizhimen South St, Beijing 100044, China

Correspondence should be addressed to Lihui Wei; weilh@bjmu.edu.cn

Received 21 May 2022; Revised 4 July 2022; Accepted 13 July 2022; Published 27 July 2022

Academic Editor: José Eleutério

Objective. This study evaluated the distribution of vaginal microbiota dysbiosis and the association with HPV viral load test in high-risk HPV-positive women before and after 50 years old. *Methods*. For this cross-sectional study, 388 HPV-positive women prior to referral to colposcopy in Peking University Peoples' Hospital were included and classified as younger than 50 years ($n = 307$) and aged 50 years or older ($n = 81$), midvagina bacterial community composition was characterized by FlashDetect™ MAX vaginal microbe detection kit, and BMRT-HPV reported type-specific viral loads/10,000 cells. *Results*. The community state type (CST) IV was the most common CST occurring in 148 women (38.1%). The proportion of CST IV in those aged 50 years or older was significantly higher than those younger than 50 years (women) (66.7% vs. 30.6%); the difference was statistically significant (<0.001). CST distribution has no statistical difference in different grades of cervical lesion, regardless of the age ($p = 0.238$ and 0.263). However, the women with high-grade cervical lesion presented a more complicated trend and the abundance of vaginal microbiota dysbiosis than low-grade lesion. HPV16/18 viral load was found to be significantly higher in CST III and CST IV than CST I/II/V ($p < 0.05$)in women younger than 50 years. *Conclusions*. In women younger than 50 years, higher HPV16/18 load was more closely associated with CST IV; however, it had no significant correlation in women aged 50 years or older.

1. Introduction

Persistent infection with high-risk human papillomavirus (hrHPV) is the main cause for the development of cervical intraepithelial neoplasia (CIN) and cervical cancer [1, 2]. Despite the controversy of the association between high viral load and the severity of cervical lesions [3, 4], it has been reported recently that HPV viral load increased with the grade of cervical lesions and is as sensitive as Cobas4800 for primary cervical cancer screening [5].

It is still unclear why some hrHPV infections resolve clinically while others persist and cause dysplasia and even cervical cancer. Many factors such as age, smoking, HIV infection, and oral contraceptives are associated with persistent HPV infection [6]. In addition to these variables, it has been proposed that the vaginal microbiota plays an important role in the development of HPV infection leading to cervical neoplasm [7]. Some vaginal microorganisms, such as *Gardnerella*, *Atopobium*, *Enterococcus*, *Streptococcus*, *Fusobacteria*, and *Mycoplasma*, as well as a decrease in the proportion of *Lactobacillus* spp., have been linked to dysbiosis that would generate an unstable microenvironment, which in turn could enable the effect of key risk factors in cervical cancer [8, 9]. However, it has been reported with greater evidence that the postmenopausal women had lower abundance of Lactobacillus species, and a more diverse community of vaginal microbes, which attributed to decreased estrogen, reduced glycogen content in vaginal epithelial cells and limits the energy source for lactobacilli [10].

To determine whether the composition of vaginal microbiota and HPV load differs before and after 50 years old, we conducted a pilot analysis of the association between vaginal microbiota, HPV viral load, and the risk of high-grade cervical lesion in different aged HPV-positive women.

2. Materials and Methods

2.1. Study Design. A total of 388 women with hrHPV positive results prior to referral to colposcopy in Peking University People's Hospital were invited to participate in this study from Nov. 2020 to Nov. 2021. The study was approved by the Ethics Committee of Peking University People's University (No. 2020PHB298-01), and written informed consent was obtained from all participants after providing detailed information about the study and its characteristics. These women were divided into the <50-year age group ($n = 307$) and ≥50-year-old group ($n = 81$), according to the previous report, which has been showed that the threshold of 50 years distinguished the peaks associated with very low lactobacilli-dominated microbiota community type and bacterial compositions dominate the cervicovaginal microbiota in women aged 50 years or older [11]. Participants who had acute vaginitis or cervicitis and vaginal antimicrobials or received estrogen replacement therapy, had sexual intercourse within 72 hours, are pregnant or up to 2 months postpartum, had a history of hysterectomy, or had immune system disease were excluded from the study. The sample collection was completed before colposcopy procedure, a vaginal speculum was placed to expose the cervix, then cervical exfoliated cell sample at the squamocolumnar junction of the cervix was obtained with a sampling brush, and then the brush was placed into a 20 mL PreservCyt1 solution (Hologic, Marlborough Mass, USA) for testing. The samples were stored at 4°C within 72 hours and at -20°C for long-term preservation. All samples were tested with cytology first, and the remaining preservation solution was used for HPV genotype, viral load, and vaginal microbes, respectively. Pathologic confirmation was performed by colposcopy-directed punch biopsy for all participants. Cervical biopsies under colposcopy were then histologically examined and classified according to the 2014 World Health Organization (WHO) Classification of Tumors of the Female Genital Tract, namely, low-grade squamous intraepithelial lesion (LSIL/CIN1) and high-grade squamous intraepithelial lesion (HSIL/CIN2 and HSIL/CIN3) [12].

2.2. BMRT HPV PCR Assay. The BMRT is detected based on PCR-based high-risk HPV assay, which was performed with the fluorescence-based multiplex HPV DNA genotyping kit (Bioperfectus Ltd., Jiangsu, P.R. China). PCR primers and corresponding TaqMan probes were developed for the 21 most prevalent HPV types to amplify the HPV L1 gene, including 14 hrHPV genotype (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and 7 medium-risk and low-risk HPV genotypes (HPV26, 53, 82, 73, 6, 11, and 81). For this study, only hrHPV was used in the statistical analysis of this study. A single-copy gene encoding DNA topoisomerase III (human TOP3) was amplified in the reaction to control DNA quality and determine the relative viral copy numbers in the samples. The normalization of HPV type-specific viral loads was performed as follows: viral load = log 10[Cn HPV/Cn TOP3 × 10,000] copies/10,000 cells, where Cn HPV is the quantity of HPV DNA and Cn TOP3 is the number of human cells. The experimental procedure was conducted according to the kit manufacturer's instructions, the detailed process was described by Dong et al. and Duan et al. [5, 13].

2.3. Vaginal Microbes Detect. Nucleic acid of vaginal microbes was extracted from 0.5 mL of vaginal swab samples using a modified TIANamp Virus DNA/RNA kit (Tiangen, China). Samples were lysed and homogenized twice with 0.2 mL lysis buffer GA and 0.3 g zirconia beads for 2 minutes by Biospec MiniBeadbeater-16 in 2 mL tube. The supernatant was treated with 40 mL protease K and 0.4 mL carrier RNA working solution at 56°C for 15 min in a 1.5 mL tube. 500 mL of ethanol was then added and mixed for 15 sec and placed for 5 min at room temperature. Sample solution was transferred into RNase-free column CR2 set and centrifuged 6,000 g for 1 min. The column CR2 was washed with 0.5 mL of buffer GD, 0.6 mL of buffer PW twice, and 500 mL of ethanol by centrifuging 6,000 g for 1 minute in turn. The column CR2 was dry and centrifuged 13,400 g for 3 min. The nucleic acid was eluted with 50 mL RNase-free ddH2O.

Vaginal microbes were detected using FlashDetect MAX vaginal microbe detection kit (Coyote, Beijing, China) in which detection and quantitation of target sequence from vaginal microbes are associated with bacterial vaginosis, aerobic vaginitis, vulvovaginal candidiasis, trichomoniasis, chlamydia, mycoplasma, sexually transmitted infection, including *Lactobacillus* (*L. gasseri*, *L. crispatus*, *L. jensenii*, and *L. iners*), *Gardnerella vaginalis*, *Atopobium vaginae*, *Bacterial vaginosis-associated bacteria 1/2* (*BVAB1/2*), *Bacterial vaginosis-associated bacteria TM7* (*BVAB-TM7*), *Megasphaera spp.*, *Prevotella fragilis*, *Mobiluncus*, *Sneathia amnii*, *Candida* (*C. spp.*, *C. krusei*, and *C. glabrata*), *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Neisseria gonorrhoeae*, *Haemophilus ducreyi*, *Herpes simplex 1*, and *Herpes simplex 2*. Real-time PCR was performed in a total volume of 20 μL (2 μL of extracted nucleic acid samples, 8 μL of VM reaction mix, and 10 μL of VM enzyme mix) in a 96-well plate using a Roche 480 (Roche Applied Sciences, Mannheim, Germany). Human RNase P gene and 16S gene were used as the internal reference gene. Reactions were incubated at 95°C for 2 min, followed by 40 cycles of denaturation at 94°C for 5 seconds and annealing and extension at 60°C for 30 seconds. The detection curve of sample has a significant exponential amplification curve with the Ct value ≤ 33; the detection result is positive.

2.4. Statistical Analysis. Statistical analysis was performed using SPSS 26.0 (IBM Corp., Armonk, NY, USA). Categorical variables were presented as frequencies and percentage (histopathological results, cervical cytology, hrHPV genotype, etc.). Viral load was measured as RLU/PC ratios (RLUs) and agreed with previous specifications in the hybrid capture assay. Viral quantification data in RLUs were initially continuous measurements. RLUs were transformed into their logarithm (Log10). Quantitative data were

described by the mean ± standard deviation (age). Nonnormally distributed continuous variables presented as median and interquartile range (IQR) (all HPV viral load, HPV16/18 viral load, and hr16/18 viral load (Log10)). Differences in frequencies for categorical variables between cases and controls were evaluated using the chi-square statistic with Yates correction. The p values for community state types (CSTs), microbiome composition, and cervical lesion were calculated using a χ^2 test. Abundance patterns within each age group and each microbiota community type were clustered by a hierarchical clustering algorithm via Ward's method. The patterns were scaled column-wise. The species selected for the heatmaps correspond to five types of CST.

3. Results

3.1. Characteristics of the Study Population. A total of 388 women were enrolled into the study and classified into <50-year group ($n = 307$) and ≥50-year group (81). The characteristics of each group are shown in Table 1. The media age was 41.5 (±10.7) (range: 19-77). 93.8% of women aged ≥50 years and 1.9% of women aged <50 years were postmenopausal; the difference was statistically significant. There were no significant differences in age, cytology, HPV status, HPV load, and the degree of cervical lesion between the two groups ($p > 0.05$). About the CST distribution, CST IV was the most common type occurring in 148 women (38.1%), CST III accounted for 35.6% and took the second place. While CST II and V accounted for the least (1.3% and 2.6%). The proportion of CST I and III in those younger than 50 years was significantly higher than those aged 50 years or older women. However, the proportion of CSTIV in those aged 50 years or older was significantly higher than those younger than 50 years women (66.7% vs. 30.6%); the difference was statistically significant (<0.001).

3.2. Characteristics of Vaginal Community State Types (CSTs) for Different Groups. Hierarchical clustering of the cervical microbiota based on the type and relative abundances of the bacterial taxa revealed that all samples clustered into five major groups: CST I, CST II, CST III, CST IV, and CST V (Figure 1). CST I was dominated by L crispatus and found in 87 women (22.4%). CST II was dominated by *L. gasseri* and present in only five women (1.3%). CST III and CST V were dominated by *L. iners* (35.6%) and *L. jensenii* (2.6%), respectively. CST IV was characterized by a diverse and complex array of facultative and strictly anaerobic BV-associated bacteria (*Gardnerella*, *Atopobium*, *BVAB_1/2*, *Enterococcus*, *Streptococcus*, *Ureaplasma*, *Prevotella*, Megasphaera, *Sneathia*, et al. and very low numbers of *Lactobacillus*) and mostly dominated in the older than 50 age group. The proportions of CSTs in different stage of cervical lesion are shown in Table 2. Although the proportions of CST IV were gradually augmented with the progression of the severity of CINs (CIN3 : 40.4% > CIN2 : 23.4% > CIN1 : 13.8% > normal : 12.8%) in women younger than 50 years, but there was a significant decrease in cervical cancer and adenocarcinoma in situ (AIS); there has no significant difference in both age group ($p = 0.238$ and 0.263). In women older than 50 years, CST I, CST II, and CST V decreased significantly, while CST III and CST IV were dominant in cervical cancer and AIS.

3.3. Identification and Cluster Analysis of Vaginal Microbiota in Different Group. As Figure 2 shown, in the CST of patients with histopathologically confirmed ≤LSIL group, the predominant type of bacteria was Gardnerella vaginalis. The structure of cervicovaginal microbiota of ≤LSIL individuals is relatively single. Gardnerella vaginalis occupy the main composition with 16.1% vs. 32.8% in <50-year- and ≥50-year-old women, respectively. However, the proportion of *Gardnerella vaginalis* was gradually reduced in histopathologically confirmed ≥HSIL group, compared with ≤LSIL individuals (≥HSIL: 14.5% vs. ≤16.1% in<50-year-old women; ≥HSIL:16.4% vs. ≤32.8% in ≥50-year-old women). Besides, the women with HSIL presented a complicated trend and the abundance of *Gardnerella vaginalis* (14.5%), *Atopobium vaginae* (5.4%), and *Enterococcus faecalis* (2.1%) was the predominant bacteria type in <50-year-old women. ≥50-year-old women diagnosed with HSIL exhibited abundant *Gardnerella vaginalis* (16.4%), *BVAB 1/2*(9.7%), and *Atopobium vaginae* (8.3%).

3.4. The Relationship of CSTs and HPV Viral Load. In women younger than 50-year-old group, there was no statistical significance between the CST type and all HPV viral load ($p > 0.05$). HPV16/18 viral load was found to be significantly higher in CST III and CST IV than CST I/II/V ($p < 0.05$). However, in women aged 50-year-or-older group, there was no significant difference in HPV viral load regardless genotype among CST types, as shown in Figure 3.

4. Discussion

HPV persistent infection leads to changes in the cervical microenvironment. It has been reported that the risk of high-grade CIN was dependent on both the HPV genotype and viral load, especially for HPV16 [2]. Besides, many studies suggest that the cervicovaginal microbiota changes are closely related to HPV infection, persistence of infection, and HSIL [14], but the relationship with the HPV viral load was unclear. Menopausal women were more likely to develop HPV persistent infection; the possible reason was the decreased estrogen levels after menopause, leading to changes in cervicovaginal microbiota, which lost its protection against pathogens including HPV [15].

Community state type (CST) was introduced first by Ravel in 2011 [16], which was used to describe the vaginal microbiota status and was divided into five groups: CST I, CST II, and CST V were normal vaginal microbiota, dominated by *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*, respectively. CST III type, dominated by *Lactobacillus iners*, represented the subhealth state of vaginal microbiota, which was most likely to transform to CST IV type, whereas the fifth CST IV has lower proportions of *Lactobacillus* spp. and higher proportions of anaerobic organisms including *Mobiluncus* spp. and *Atopobium vaginae*.

Table 1: Characteristics of 388 HPV-positive women in different age distribution (*n* (%)).

	<50 *n* = 307	≥50 *n* = 81	*p* value
Age			
n, mean ± SD	41.5 (±10.7)		
Menopause status	6 (1.9)	76 (93.8)	<0.001
Biopsy			
Normal	34 (11.1)	14 (17.3)	
LSIL/CIN1	38 (12.4)	9 (11.1)	
HSIL/CIN2	86 (28.0)	19 (23.5)	0.452
HSIL/CIN3	122 (39.7)	29 (35.8)	
SCC/AIS	27 (8.8)	10 (12.3)	
Cytology			
NILM	160 (52.1)	42 (51.9)	
ASCUS/LSIL	69 (22.5)	14 (17.3)	0.849
ASC-H+	78 (25.4)	25 (30.7)	
CST type			
I	81 (26.4)	6 (7.4)	
II	3 (1.0)	2 (2.5)	
III	120 (39.1)	18 (22.2)	<0.001
IV	94 (30.6)	54 (66.7)	
V	9 (2.9)	1 (1.2)	
HPV viral load			
Log (all); *n*, median (IQR)	4.79 (3.79-5.68)	4.60 (3.37-5.75)	0.436
Log (16/18); *n*, median (IQR)	1.44 (0-4.82)	1.52 (0-4.44)	0.556
Log (hrHPV); *n*, median (IQR)	4.78 (3.68-5.66)	4.52 (3.30-5.72)	0.365
Multiple HPV types			
Yes	90 (29.3)	30 (37.0)	0.181
No	217 (70.7)	51 (63.0)	
HR-HPV			
HPV16/18(+)	142 (47.5)	36 (46.2)	0.833
Other HR(+)	157 (52.5)	42 (53.8)	

LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma; AIS: adenocarcinoma in situ; NILM: negative for intraepithelial lesions or malignancy; ASCUS: atypical squamous cells of undetermined significance ASC-H: atypical squamous cell-cannot exclude HSIL; CST: community state types.

In this study, in all the HPV-infected women, cervicovaginal microbiota were dominated by CST IV (38.1%) and CST III (35.6%); just as Chen et al. [17] reported that the most dominant CST in the HPV positive groups (HPV, LSIL, HSIL, and cancer) was CST IV, while CST II and CST IV accounted for the least in Chinese HPV positive women. It has been reported that Lactobacillus dominated the cervicovaginal microbiota of health and HPV transient infection women, while the microbiota diversity of HPV persistent infection patients significantly increased and the proportion of anaerobic bacteria in the composition significantly increased [15]. Although the healthy women were not included in our study, we found CST IV was more dominant in women aged 50 years or older (66.7%) than in women younger than 50 years (30.6%) when stratified by different age thresholds. Although postmenopausal women were less likely to have a CST dominated by Lactobacillus spp. than premenopausal women, nearly 50% of postmenopausal women have a high relative abundance of Lactobacillus spp. [10], and the presence of Lactobacillus spp. has been associated with exogenous hormone use [18]. In our study, this rate was higher, which may be related to the fact that all the participants were HPV-positive and exogenous estrogens were excluded. Burton et al. [19] reported *Atopobium vaginae* as a common member of the vaginal microbiota of postmenopausal women, and Brotman et al. found a distinct bacterial community state (CST IV-A) with low relative abundance of *Lactobacillus* was associated with vulvovaginal atrophy [20]. After menopause, the composition of vaginal microbiota has been shown to be less likely dominated by Lactobacillus spp. and more likely to be composed predominantly of anaerobic and aerobic bacteria [20].

Besides, our result showed that a proportion of CST IV was gradually increased with the progression of CIN severity, but decreased in cervical cancer, although there was no significant difference, which is different from the Chen et al.'s

Figure 1: Vaginal microbiome composition according to age, HPV status, cervical lesion, and vaginal community state types.

findings that the proportions of CST IV were gradually augmented with the progression of the severity of CINs (cancer > HSIL > LSIL) [17]. However, CST III and CST IV, not CST I, II, and V, were dominant in cervical cancer and AIS, especially in women aged 50 years or older.

Besides, it has been reported that the vaginal microbiota differences were primarily attributed to HPV infection (or subtype) and not SILs, indicating that infection itself may lead to changes in the vaginal microbial community [21].

It has been reported that HPV infection increased vaginal bacterial richness and diversity regardless of the status of CINs [17]. About CIN, there was a research finding that *Pseudomonas stutzeri*, *Bacteroides fragilis*, *Lactobacillus delbrueckii*, *Atopobium vaginae*, and *Streptococcus agalactiae* were associated with CIN status [22]. Wu et al. found that *Delftia genus* might be a microbiological hallmark of cervical lesion [23] In our study, we found that the predominant type of bacteria younger than 50 years was Gardnerella vaginalis, *Atopobium vaginae*, and *Enterococcus faecalis* regardless of low-grade or high-grade CIN. Among the women aged 50 years or older, except the *Gardnerella vaginalis* and *Atopobium vaginae*, *Streptococcus agalactiae* and *BVAB* 1/2 were the predominant types in the ≤LSIL group and the ≥HSIL group, respectively, further suggesting that the vaginal

Table 2: The proportions of CSTs in different stage of histopathologically confirmed cervical lesions (n (%)).

	Normal	LSIL/CIN1	HSIL/CIN2	HSIL/CIN3	SCC/AIS	p value
Age < 50						
CST I, CST II, CST V	8 (8.6)	9 (9.7)	36 (38.7)	36 (38.7)	4 (4.3)	
CST III	14 (11.7)	16 (13.3)	28 (23.3)	48 (40.0)	14 (11.7)	0.238
CST IV	12 (12.8)	13 (13.8)	22 (23.4)	38 (40.4)	9 (9.6)	
Age ≥ 50						
CST I, CST II, CST V	1 (11.1)	1 (11.1)	3 (33.3)	4 (44.4)	0 (0.0)	
CST III	2 (11.1)	4 (22.2)	3 (16.7)	4 (22.2)	5 (27.8)	0.263
CST IV	11 (20.4)	4 (7.4)	13 (24.1)	21 (38.9)	5 (9.3)	

CST: community state types; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma; AIS: adenocarcinoma in situ.

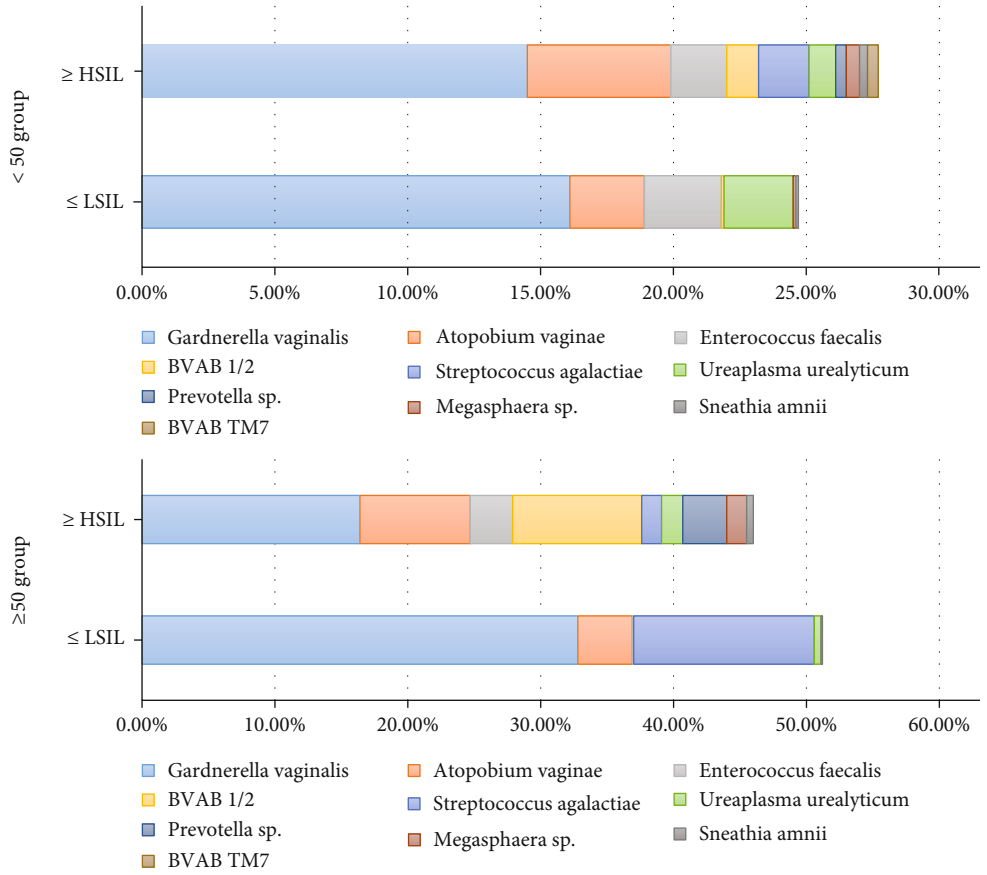
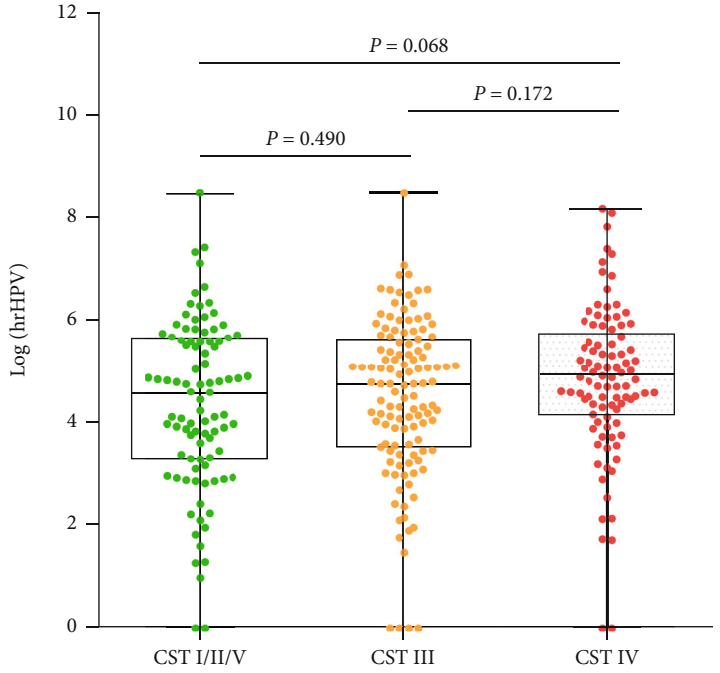


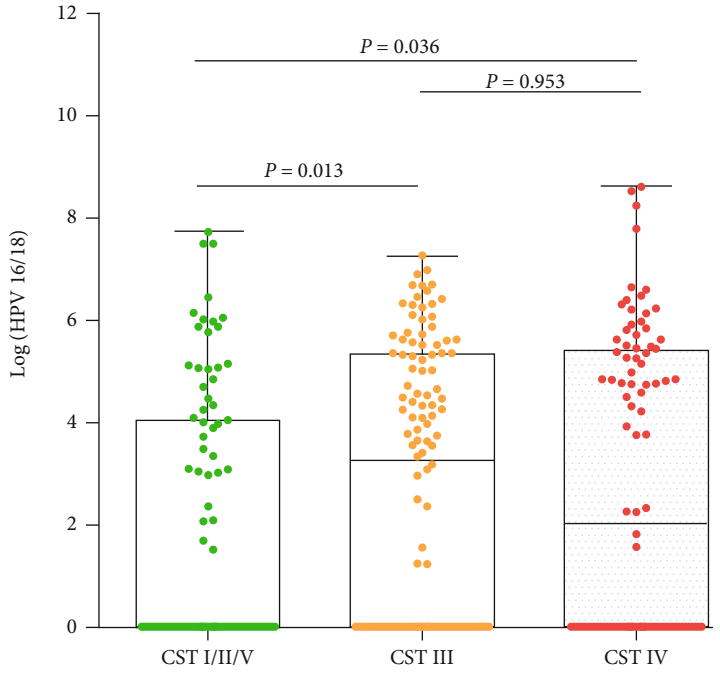
Figure 2: Average relative abundances of the 10 prevalent genera in vaginal microbiota in different degree of cervical lesion of different age. Note: ≥HSIL including histopathologically confirmed HSIL/CIN2, HSIL/CIN3, SCC, and AIS; ≤LSIL including histopathologically confirmed LSIL/CIN1 and normal.

microbiome of HPV-infected women was associated with different ages. We also found that the abundance of *Gardnerella vaginalis* was gradually reduced and bacterial diversity increased with the progression of CINs severity, which was consistent with the previous report [17, 24]. However, the ≥HSIL group presented a complicated trend and the abundance of vaginal microbiota diversity than the ≤LSIL group; this is different from some other studies reporting that there was no association between the diversity of vaginal microbiome and the CIN progression [25, 26]. Over all, in our study, *Gardnerella vaginalis* and *Atopobium vaginae* dominated the cervicovaginal microbiota in both LSIL and HSIL+ women, which were thought to be associated with bacterial vaginosis (BV) [27] and had a higher CIN risk [28].

Besides, one of the key findings of our study was that HPV16/18 viral load was found to be significantly higher in CST III and CST IV in women younger than 50 years old; however, there was no connection between CST type and HPV viral load in women older than 50 years old. An association between hrHPV viral load in cervical samples



(a)



(b)

Figure 3: Continued.

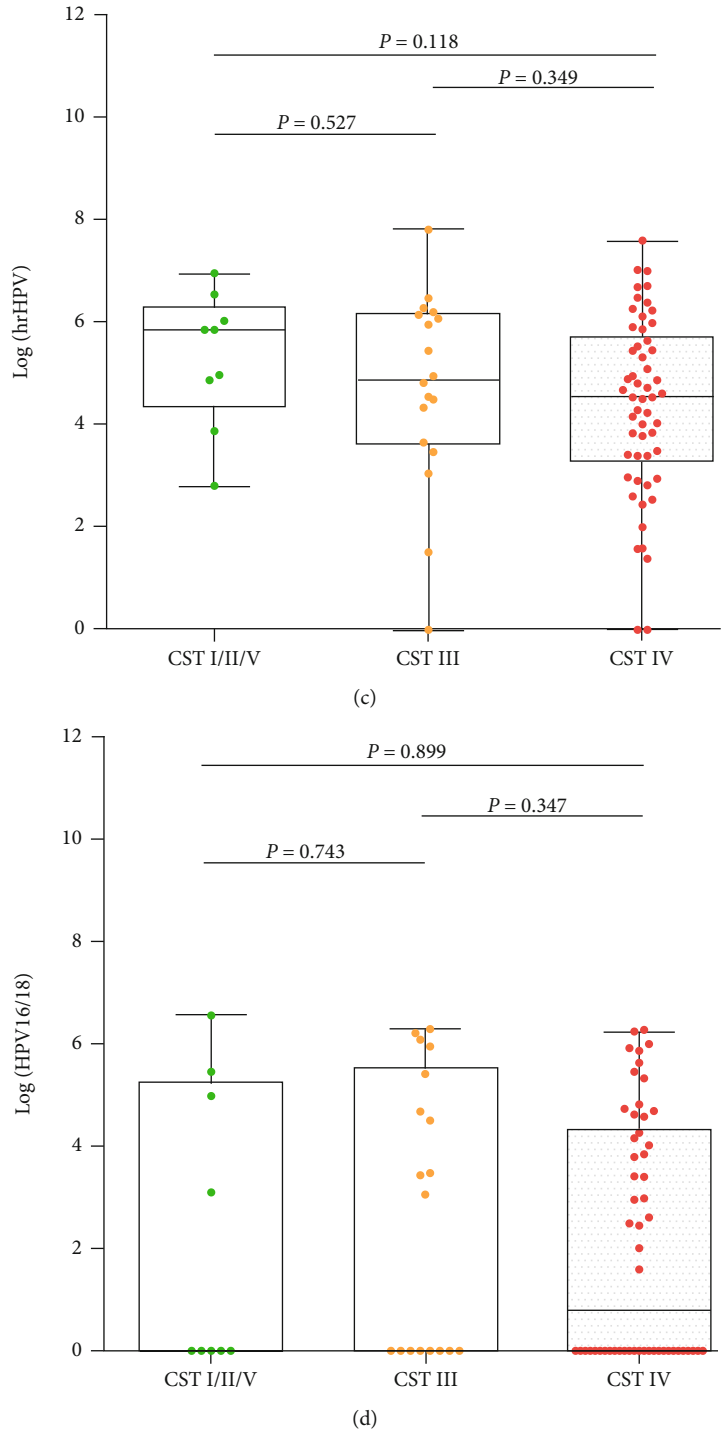


Figure 3: HPV viral load for different vaginal CSTs. (a) hrHPV load < 50 years old; (b) HPV16/18 load < 50 years old; (c) hrHPV load ≥ 50 years old; (d) HPV16/18 load ≥ 50 years old.

and severity of prevalent cervical disease was first described in 1999 [29] and replicated in numerous studies [30–32] and proved that HPV16/18 was more sensitive and specific than 12 other subtypes of hrHPV, suggesting that HPV viral load is a type-specific biological indicator of cervical cancer [33]. It has been approved that a microbial environment with a higher proportion of anaerobic bacteria and a lower proportion of *Lactobacillus* spp. is more likely to HPV infection, and CST IV was related with an increased risk of transitioning to an HPV positive state [34, 35]. Besides, some studies identified cervicovaginal microbiota dysbiosis closely related to HPV persistent infection, and transition between clusters was more frequent in women with persistent HPV16 infection (34%) than in women with transient infection (19%) [15, 36] Based on the relationship with high HPV16/18 viral load in our study, CST IV has an enhanced

effect on the risk of cervical high-grade lesions in women younger than 50 years old. However, for women over 50 years of age, vaginal microbiota dysbiosis is more common regardless normal or cervical lesions, as evidenced by the high proportion of CST IV in this study, so there is no significant association with HPV viral load among this population.

The strength of this study is that it is stratified by age to interpret the vaginal microbial compositions of HPV-positive women with different stages of cervical lesion and the relationship with HPV viral load, which has not yet been well elucidated. The limitations of this study were that it was a cross-sectional study. Hence, we could not conclude any causal relationship between the vaginal microbiome and HPV viral load or CIN diseases. We have to conduct longitudinal studies to study relationships between the dynamics of the vaginal microbiome and the HPV viral load and the progression or remission of CIN diseases. In addition, the underlying biological mechanisms also need to be detailed. Finally, we lack information about covariates that could affect the vaginal microbiota, such as status of smoking, menstrual cycle, sexual behavior, and hormonal contraceptives use [37, 38]. However, previous researches have shown small or no significant effects of the menstrual cycle stage or of use of hormonal contraceptives on the vaginal microbiota [39, 40]. Sexual behavior has also been shown to affect the vaginal microbiota [41]; therefore, we try to avoid the influence of this factor on population enrollment in this study.

5. Conclusion

By analyzing the vaginal microbial distribution of HPV infected people of different ages triaged by 50 years, our study found that women aged 50 years or older had higher type IV distribution and had no significant correlation with HPV load; However, higher HPV16/18 load was more closely associated with CST IV in women younger than 50 years.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Peking University People's Hospital Ethics Committee (2020PHB298-01).

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors have no relevant financial or nonfinancial interests to disclose.

Authors' Contributions

Data collection, study conception, and design the study were performed by Mingzhu Li. Personnel organization and data collection were performed by Chao Zhao. Sample collection was performed by Yun Zhao and Jingran Li. Supervision of the research program and manuscript review and guidance during the study were performed by Lihui Wei. The first draft of the manuscript was written by Mingzhu Li and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Mingzhu Li and Chao Zhao have contributed equally to this work and share the first authorship.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2021YFC2701200 and 2021YFC2701202) and the Research and Development Fund of Peking University People's Hospital (RDL2020-02). Thanks are due to Coyote Co., Ltd. that provided the testing kits for the project and also due to Ching-Tung Yeh for giving guidance and advice on statistics and interpretation of data.

References

[1] M. Schiffman, J. Doorbar, N. Wentzensen, S. de Sanjose, and C. Fakhry, "Carcinogenic human papillomavirus infection," *Nature reviews Disease primers*, vol. 2, no. 1, p. 16086, 2016.

[2] R. Adcock, J. Cuzick, W. C. Hunt et al., "Role of HPV genotype, multiple infections, and viral load on the risk of high-grade cervical neoplasia," *Cancer Epidemiology, Biomarkers & Prevention*, vol. 28, no. 11, pp. 1816–1824, 2019.

[3] S. Andersson, H. Safari, M. Mints, I. Lewensohn-Fuchs, U. Gyllensten, and B. Johansson, "Type distribution, viral load and integration status of high-risk human papillomaviruses in pre-stages of cervical cancer (CIN)," *British Journal of Cancer*, vol. 92, no. 12, pp. 2195–2200, 2005.

[4] V. Dalstein, D. Riethmuller, J. L. Pretet, C. K. Le Bail, and J. L. Sautiere, "Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study," *International Journal of Cancer*, vol. 106, no. 3, pp. 396–403, 2003.

[5] L. Duan, H. Du, C. Wang et al., "The application of BMRT-HPV viral load to secondary screening strategies for cervical cancer," *Plos One*, vol. 15, no. 5, article e232117, 2020.

[6] A. Mitra, D. A. MacIntyre, J. R. Marchesi, Y. S. Lee, P. R. Bennett, and M. Kyrgiou, "The vaginal microbiota, human papillomavirus infection and cervical intraepithelial neoplasia: what do we know and where are we going next?," *Microbiome*, vol. 4, no. 1, p. 58, 2016.

[7] A. Audirac-Chalifour, K. Torres-Poveda, M. Bahena-Román et al., "Cervical microbiome and cytokine profile at various stages of cervical cancer: a pilot study," *Plos One*, vol. 11, no. 4, article e153274, 2016.

[8] S. M. Kovachev, "Cervical cancer and vaginal microbiota changes," *Archives of Microbiology*, vol. 202, no. 2, pp. 323–327, 2020.

[9] W. Ritu, W. Enqi, S. Zheng, J. Wang, Y. Ling, and Y. Wang, "Evaluation of the associations between cervical microbiota and HPV infection, clearance, and persistence in cytologically normal women," *Cancer Prevention Research*, vol. 12, no. 1, pp. 43–56, 2019.

[10] R. Hummelen, J. M. Macklaim, J. E. Bisanz et al., "Vaginal microbiome and epithelial gene array in post-menopausal women with moderate to severe dryness," *Plos One*, vol. 6, no. 11, article e26602, 2011.

[11] N. R. Nene, D. Reisel, A. Leimbach, D. Franchi, and A. Jones, "Association between the cervicovaginal microbiome, BRCA1 mutation status, and risk of ovarian cancer: a case-control study," *The Lancet Oncology*, vol. 20, no. 8, pp. 1171–1182, 2019.

[12] O. Reich, S. Regauer, C. Marth et al., "t1Precancerous lesions of the cervix, vulva and vagina according to the 2014 WHO classification of tumors of the female genital tract," *Geburtshilfe und Frauenheilkunde*, vol. 75, no. 10, pp. 1018–1020, 2015.

[13] B. Dong, P. Sun, G. Ruan et al., "Type-specific high-risk human papillomavirus viral load as a viable triage indicator for high-grade squamous intraepithelial lesion: a nested case-control study," *Cancer management and research*, vol. 10, pp. 4839–4851, 2018.

[14] L. Anton, L. J. Sierra, A. DeVine et al., "Common cervicovaginal microbial supernatants alter cervical epithelial function: mechanisms by which Lactobacillus crispatus contributes to cervical health," *Frontiers in microbiology*, vol. 9, p. 2181, 2018.

[15] B. Qingqing, Z. Jie, Q. Songben, C. Juan, Z. Lei, and X. Mu, "Cervicovaginal microbiota dysbiosis correlates with HPV persistent infection," *Microbial Pathogenesis*, vol. 152, p. 104617, 2021.

[16] J. Ravel, P. Gajer, Z. Abdo et al., "Vaginal microbiome of reproductive-age women," *Proceedings of the National Academy of Sciences*, vol. 108, Suppl 1, pp. 4680–4687, 2011.

[17] Y. Chen, X. Qiu, W. Wang et al., "Human papillomavirus infection and cervical intraepithelial neoplasia progression are associated with increased vaginal microbiome diversity in a Chinese cohort," *BMC infectious diseases*, vol. 20, no. 1, p. 629, 2020.

[18] W. L. Pabich, S. D. Fihn, W. E. Stamm, D. Scholes, E. J. Boyko, and K. Gupta, "Prevalence and determinants of vaginal flora alterations in postmenopausal women," *The Journal of Infectious Diseases*, vol. 188, no. 7, pp. 1054–1058, 2003.

[19] J. P. Burton, E. Devillard, P. A. Cadieux, J. A. Hammond, and G. Reid, "Detection of Atopobium vaginae in postmenopausal women by cultivation-independent methods warrants further investigation," *Journal of Clinical Microbiology*, vol. 42, no. 4, pp. 1829–1831, 2004.

[20] R. M. Brotman, M. D. Shardell, P. Gajer et al., "Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy," *Menopause*, vol. 25, no. 11, pp. 1321–1330, 2018.

[21] M. E. Nieves-Ramírez, O. Partida-Rodríguez, P. Moran et al., "Cervical squamous intraepithelial lesions are associated with differences in the vaginal microbiota of Mexican women," *Microbiology Spectrum*, vol. 9, no. 2, article e14321, 2021.

[22] C. Zhang, Y. Liu, W. Gao et al., "The direct and indirect association of cervical microbiota with the risk of cervical intraepithelial neoplasia," *Cancer Medicine*, vol. 7, no. 5, pp. 2172–2179, 2018.

[23] M. Wu, J. Gao, Y. Wu et al., "Characterization of vaginal microbiota in Chinese women with cervical squamous intraepithelial neoplasia," *International Journal of Gynecological Cancer*, vol. 30, no. 10, pp. 1500–1504, 2020.

[24] A. Mitra, D. A. MacIntyre, Y. S. Lee et al., "Cervical intraepithelial neoplasia disease progression is associated with increased vaginal microbiome diversity," *Scientific reports*, vol. 5, article 16865, 2015.

[25] X. Huang, C. Li, F. Li, J. Zhao, X. Wan, and K. Wang, "Cervicovaginal microbiota composition correlates with the acquisition of high-risk human papillomavirus types," *International Journal of Cancer*, vol. 143, no. 3, pp. 621–634, 2018.

[26] C. J. Piyathilake, N. J. Ollberding, R. Kumar, M. Macaluso, R. D. Alvarez, and C. D. Morrow, "Cervical microbiota associated with higher grade cervical intraepithelial neoplasia in women infected with high-risk human papillomaviruses," *Cancer Prevention Research*, vol. 9, no. 5, pp. 357–366, 2016.

[27] J. P. Menard, F. Fenollar, M. Henry, F. Bretelle, and D. Raoult, "Molecular quantification of Gardnerella vaginalis and Atopobium vaginae loads to predict bacterial vaginosis," *Clinical Infectious Diseases*, vol. 47, no. 1, pp. 33–43, 2008.

[28] H. Tuominen, S. Rautava, S. Syrjanen, M. C. Collado, and J. Rautava, "HPV infection and bacterial microbiota in the placenta, uterine cervix and oral mucosa," *Scientific Reports*, vol. 8, no. 1, p. 9787, 2018.

[29] D. C. Swan, R. A. Tucker, G. Tortolero-Luna et al., "Human papillomavirus (HPV) DNA copy number is dependent on grade of cervical disease and HPV type," *Journal of Clinical Microbiology*, vol. 37, no. 4, pp. 1030–1034, 1999.

[30] D. M. Hernandez-Hernandez, L. Ornelas-Bernal, M. Guido-Jimenez, T. Apresa-Garcia, and I. Alvarado-Cabrero, "Association between high-risk human papillomavirus DNA load and precursor lesions of cervical cancer in Mexican women," *Gynecologic Oncology*, vol. 90, no. 2, pp. 310–317, 2003.

[31] Z. Wu, Y. Qin, L. Yu et al., "Association between human papillomavirus (HPV) 16, HPV18, and other HR-HPV viral load and the histological classification of cervical lesions: results from a large-scale cross-sectional study," *Journal of Medical Virology*, vol. 89, no. 3, pp. 535–541, 2017.

[32] A. Manawapat-Klopfer, L. Wang, J. Haedicke-Jarboui et al., "HPV16 viral load and physical state measurement as a potential immediate triage strategy for HR-HPV-infected women: a study in 644 women with single HPV16 infections," *American Journal of Cancer Research*, vol. 8, no. 4, pp. 715–722, 2018.

[33] M. Wang, B. Hou, X. Wang et al., "Diagnostic value of high-risk human papillomavirus viral load on cervical lesion assessment and ASCUS triage," *Cancer Medicine*, vol. 10, no. 7, pp. 2482–2488, 2021.

[34] J. E. Lee, S. Lee, H. Lee et al., "Association of the vaginal microbiota with human papillomavirus infection in a Korean twin cohort," *Plos One*, vol. 8, no. 5, article e63514, 2013.

[35] E. M. Jalil, F. I. Bastos, P. P. Melli et al., "HPV clearance in postpartum period of HIV-positive and negative women: a prospective follow-up study," *BMC infectious diseases*, vol. 13, p. 564, 2013.

[36] M. Berggrund, I. Gustavsson, R. Aarnio et al., "Temporal changes in the vaginal microbiota in self-samples and its

association with persistent HPV16 infection and CIN2," *Virology journal*, vol. 17, no. 1, p. 147, 2020.

[37] S. B. Rifkin, M. R. Smith, R. M. Brotman, R. M. Gindi, and E. J. Erbelding, "Hormonal contraception and risk of bacterial vaginosis diagnosis in an observational study of women attending STD clinics in Baltimore, MD," *Contraception*, vol. 80, no. 1, pp. 63–67, 2009.

[38] T. L. Cherpes, S. L. Hillier, L. A. Meyn, J. L. Busch, and M. A. Krohn, "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 Diseases*, vol. 35, no. 1, pp. 78–83, 2008.

[39] B. Chaban, M. G. Links, T. P. Jayaprakash et al., "Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle," *Microbiome*, vol. 2, p. 23, 2014.

[40] S. L. Achilles, M. N. Austin, L. A. Meyn, F. Mhlanga, Z. M. Chirenje, and S. L. Hillier, "Impact of contraceptive initiation on vaginal microbiota," *American Journal of Obstetrics and Gynecology*, vol. 218, no. 6, pp. 622.e1–622.e10, 2018.

[41] J. M. Wessels, J. Lajoie, D. Vitali et al., "Association of high-risk sexual behaviour with diversity of the vaginal microbiota and abundance of Lactobacillus," *Plos One*, vol. 12, no. 11, article e187612, 2017.