STROBE-sequencing analysis of the vaginal microecology of 4- to 6-year-old girls in Southwest China

Yiduo Zhang, MM[a,b], Ting Liu, MD[a,b], Jingying Lin, BS[a,b], Fan Yu, MM[a,b], Zhengqiang Hu, MM[a,b,*]

Abstract

We investigated the vaginal flora diversity of preschool-aged (ie, 4–6-year-old) girls in southwest China.

Fourteen preschool-aged girls were enrolled in this study. The statuses and differences in their vaginal flora were evaluated by Gram staining, bacterial culturing, and sequencing analysis.

Gram staining and microbial culturing showed that the main vaginal flora of the preschool-aged girls were Gram-negative bacilli, whereas the main vaginal flora of healthy adult controls were large Gram-positive bacilli such as *Lactobacillus crispatus*. Shannon and Simpson indexes indicated that the bacterial diversity tended to decrease with age. The species abundance heat map showed that the vaginal microecology of the girls differed slightly at different ages but mainly comprised *Pseudomonas*, *Methylobacterium*, Sphingomona,s and *Escherichia*. The functional abundance heat map indicated that the bacterial functions increased with age.

The vaginal microecology of preschool-aged girls differs from that of adults. A comprehensive understanding of the vaginal flora diversity of preschool-aged girls will aid in clinically diagnosing vulvovaginitis in preschool-aged girls.

Abbreviations: COG = clusters of orthologous group, PBS = phosphate-buffered saline, PCR = polymerase chain reaction, SD = standard deviation, spp = species.

Keywords: preschool girls, sequencing analysis, vaginal discharge

1. Introduction

The vaginal microecology is composed of the vaginal flora, the endocrine regulatory system, and the reproductive tract and plays an indispensable role in preventing genital tract infections in women and girls.[1] The vaginal flora is composed of millions of microorganisms that are both symbiotic and antagonistic to each other.[2] A better understanding of the vaginal flora in healthy women and girls could lead to better diagnosis and treatment of vaginal diseases.

The vaginal microecology maintains a dynamic balance between the host and environmental factors.[2] In addition to demographics such as ethnicity, location, and age, several environmental factors, including temperature and humidity, affect the composition of the vaginal microbiome.[3] However, in most cases, imbalances in the vaginal microecology are closely

a Department of Laboratory Medicine, West China Second University Hospital, Sichuan University, b Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Chengdu, Sichuan, China.

* Correspondence: Zhengqiang Hu, Department of Laboratory Medicine, West China Second University Hospital, Sichuan University, No. 20, Section 3, Renmin South Road, Chengdu, Sichuan 610041, P. R. China (e-mail: huzhengqiang1234@163.com).

related to inflammation, cervical lesions, childbirth diseases and sexually transmitted diseases.[4–6] The vaginal microecology is also affected by hormone levels and immunity.[7] Additionally, hormone levels are greatly affected by age; therefore, the vaginal microecology likely differs in women of different ages.[8] To date, the vaginal microecology has been reported in women of childbearing age and premenstrual adolescent girls but not in preschool-aged (ie, 4–6-year-old) girls.[9,10] Consequently, less is known about the true vaginal flora composition in preschool-aged girls. The factors triggering vulvovaginitis in preschool-aged girls are complex and commonly include poor hygiene or nonspecific irritants, bacterial infections, and skin diseases of the vulva.[11–13] Vulvovaginitis in preschool-aged girls has been reported as nonspecific in 25% to 75% of cases.[14] This causes difficulty in diagnosing vulvovaginitis. Therefore, identifying the characteristics of the vaginal flora in healthy preschool-aged girls is valuable for clinically diagnosing vulvovaginitis in preschool-aged girls and will provide a relevant basis for studying the evolution of the vaginal flora in women and girls.

Sequencing analysis is a method of microbiological analysis that is based on second-generation sequencing technology and enables directly detecting the presence and abundance of specific species from complex community samples. Briefly, sequencing analysis uses the genome of the microorganism in the sample as the research object and then analyzes the microbial diversity, population structure, functional activity and cooperative relationships of the microorganism.[15] This technique overcomes the problem that some microorganisms are difficult or even impossible to culture.[16,17] Moreover, high-throughput sequencing can provide more precise, rapid and comprehensive information regarding vaginal microbial communities.

In this study, we analyzed the vaginal flora diversity in healthy preschool-aged girls. We also analyzed the specific composition of the vaginal microbiome and its bacterial functions. Our study will lay a foundation for a comprehensive understanding of the vaginal microecology of healthy preschool-aged girls in southwest China and provide guidance for diagnosing vulvovaginitis in preschool-aged girls.

2. Material and methods

2.1. Study subjects

The Clinical Research Ethics Committee of West China Second University Hospital, Sichuan University, approved this study. All participants or their parents provided written informed consent before the analysis (approval number 2017[25]). Fourteen healthy preschool-aged girls were enrolled at the gynecology clinic of West China Second Hospital, Sichuan University. The girls were 4 to 6 years' old (mean 5.71 ± 1.22 years) and were all Han nationalities from southwest China. Routine examination of their vaginal discharge showed no infections of *Candida* spp, *Trichomonas vaginalis*, or *Neisseria gonorrhoeae*. The microflora were mainly Gram-negative bacilli and Gram-positive cocci, which were common in the preschool-aged girls.

Fourteen healthy women of childbearing age (mean 29.33 ± 4.75 years, range 25–35 years) were enrolled as the control group. *Lactobacillus* spp were the dominant bacteria in the vaginal discharge of the control group, and no pathogenic microorganisms were found.

The inclusion criteria were: no complaints of vulvar pruritus or abnormal vaginal discharge; no *Candida* spp, *Trichomonas*

vaginalis, or Gram-negative diplococci in the vaginal discharge; no history of systemic application of antibiotics or local vaginal medication within 2 weeks before enrollment.

2.2. Routine examination

Because collecting cervical secretions would likely cause trauma to preschool-aged girls, and to avoid contamination with vulvar microorganisms during sampling, vaginal discharge from the girls was collected near the hymenal orifice.[18] For the preschool-aged girls, samples of uncontaminated vaginal discharge were collected outside the hymen. For the women, samples of uncontaminated vaginal discharge were collected from the upper one-third of the lateral wall of the vagina. Three samples were taken from each participant using sterile cotton swabs. The first sample was quickly placed in a sterile tube containing 1 ml of phosphate-buffered saline (PBS, pH 7.4) for genome isolation. The second sample was smeared evenly on a glass slide, Gram stained, and observed under $100 \times$ oil immersion for microbiome evaluation.[19] The third sample was cultured both aerobically and anaerobically.

2.3. Sequencing analysis

Total genomic DNA was extracted using a bacterial genomic DNA extraction kit (Sangon Biotech Company, Shanghai, China) and then used as a template to amplify the 400-bp sequence of the 16 s rDNA V3–V4 region. The primers were universal to the Illumina adapter: 341F (5′-CCCTACACGACGCTCTTCCGAT-CTG (barcode) CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTGGAGTTCCTTGG CACCCGAGAATTCCA GACTACHVGGGTATCTAATCC-3′). The PCR volume was 30 μL, including 15 μL 2 × Taq Master Mix, 1 μL 10 μM Bar-PCR primer F, 1 μL 10 μmol/L primer R, 10 to 20 ng genomic DNA, and H_2O added to 30 μL. The reaction conditions were 5 cycles at 94°C for 3 minutes, 94°C for 30 seconds, 45°C for 20 seconds, and 65°C for 30 seconds, and 20 cycles at 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes. After the PCR was complete, a second amplification round was performed. The total PCR reaction volume was 30 μL, including 15 μL 2 × Taq Master Mix, 1 μL 10 μmol/L Bar-PCR primer F, 1 μL 10 μ mol/L primer R, 20 ng PCR products (last round) and H_2O added to 30 μL. The reaction conditions were 5 cycles of 95°C for 3 minutes, 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes. The PCR product was purified using magnetic beads (Transgen Biotech Company, Beijing, China). The recovered DNA was accurately quantified with a Qubit3.0 DNA detection kit (Thermo Fisher Scientific Inc, Massachusetts), then sent to Sangon Biotech Company for high-throughput sequencing.

2.4. Statistical analysis

The basic data were analyzed with SPSS Statistics, ver. 25.0 (SPSS Inc, Chicago, IL). Subjects' ages and other indicators are presented as the mean ± standard deviation (SD). Colony counts are presented as the mean ± standard error of the mean (SEM). Between-group comparisons were performed using one-way analysis of variance. $P < .05$ was considered statistically significant. Sequencing data analyses were conducted using R programming language (version 3.5.1).
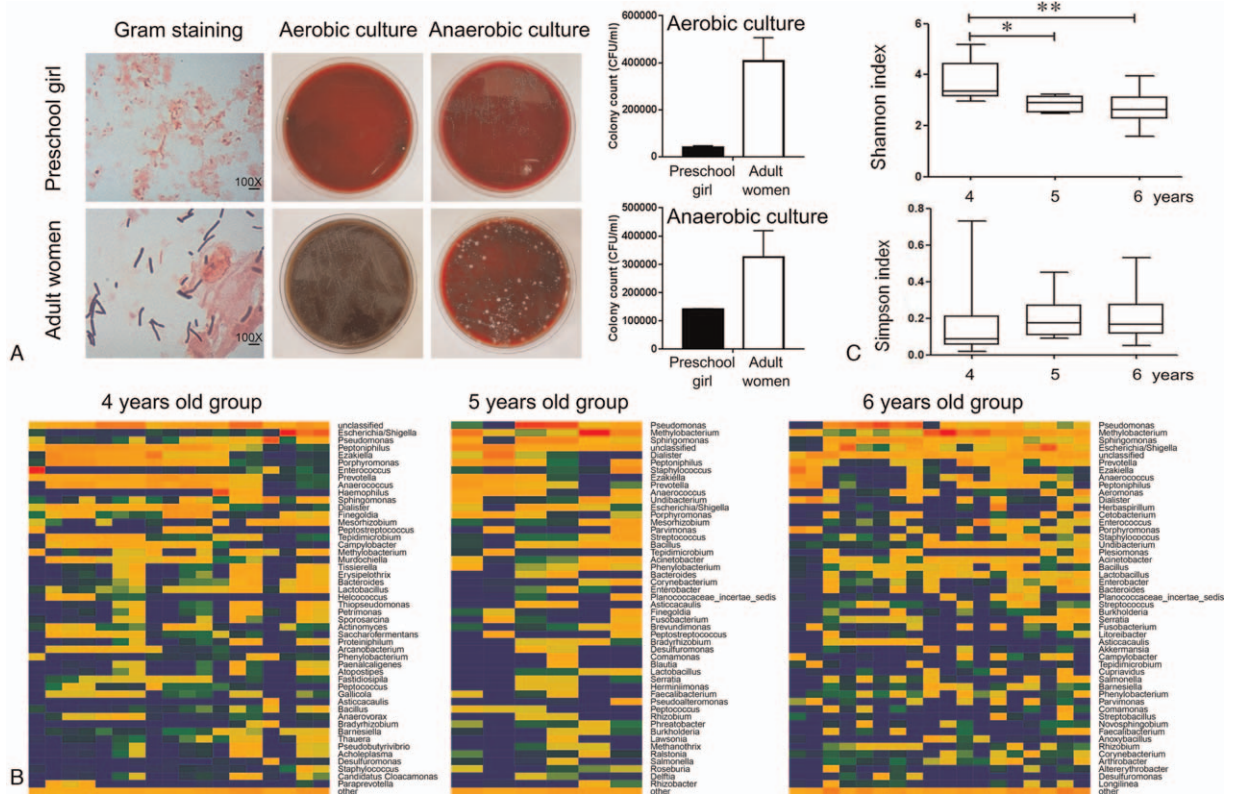
Figure 1. Diversity of the vaginal microecology in preschool-aged girls. (A) Gram staining (1000×) and microbial culturing of the vaginal discharge of healthy preschool-aged girls and women of childbearing age. (B) Analysis of species abundance heat maps for different age groups. (C) Shannon and Simpson indexes of the vaginal microecology of preschool-aged girls in different age groups. These findings suggest that the vaginal microecology of healthy preschool-aged girls differs from that of healthy women of childbearing age, and the vaginal microecology diversity is closely related to age in preschool-aged girls ($^{*}P < .05$ and $^{**}P < .01$).

3. Results

3.1. Diversity of the vaginal microecology in preschool-aged girls

Vaginal discharge from both the preschool-aged girls and the healthy women of childbearing age was smeared and stained for microscopic examination under the same conditions. Samples from the preschool-aged girls contained Gram-negative bacilli, Gram-positive cocci and Gram-positive bacilli; samples from the healthy women of childbearing age contained many Gram-positive bacilli (Fig. 1A). The vaginal discharge was also cultured both aerobically and anaerobically on blood agar plates. For the species abundance heat maps, the samples were divided into the 4-year-old, 5-year-old, and 6-year-old groups. The main flora in the vaginal discharge from the 4-year-old girls were *Peptoniphilus*, *Ezakiella*, *Porphyromonas*, *Prevotella*, *Aerococcus*, *Tibiella*, and *Campylobacter* (Fig. 1B). The main flora in the vaginal discharge of the 5-year-old girls were *Pseudomonas*, *Sphingomonas*, *Acinetobacter* and *Enterobacteria*, and those of the 6-year-old girls were *Pseudomonas*, *Sphingomonas*, *Aeromonas*, *Cetobacterium*, *Plesiomonas*, and *Burkholderia*. The Shannon index is an indicator used to estimate microbial diversity in samples and is often used to reflect the alpha diversity index, with higher Shannon index values indicating higher microbial diversity. The Simpson index is used to describe community diversity in a specific environment, and its value is inversely proportional to the microbial diversity. Shannon and Simpson index analyses of the vaginal discharge of the younger girls revealed that the microbial diversity decreased with age, the Shannon index of the vaginal discharge of 4-year-old was significantly different from that 5-year-old ($P = .035$) and 6-year-old groups ($P = .003$) (Fig. 1C). These findings suggest that the diversity and species abundances of the vaginal microecology differ among preschool-aged girls of different ages.

3.2. Community structure and bacterial functions of vaginal microorganisms in preschool-aged girls

The distribution map of the community structure of all samples at the genus level revealed some individual differences in the specimens of the 4-year-old group. Samples 7 to 9 included mainly *Escherichia coli* (Fig. 2A), which may have been due to fecal contamination or because the source of the vaginal flora in younger girls is related to intestinal microorganisms; however, this theory remains to be verified. Studying the functional abundance heatmap of the clusters of orthologous groups (COGs) revealed that the bacterial functions were continuously changing in the different age groups (Fig. 2B). In the 4-year-olds, the vaginal bacterial colonies played a less physiological role. However, the bacterial colony functions increased significantly in the 5- and 6-year-old groups. This was mainly reflected in energy production and conversion, amino acid transport and metabo-
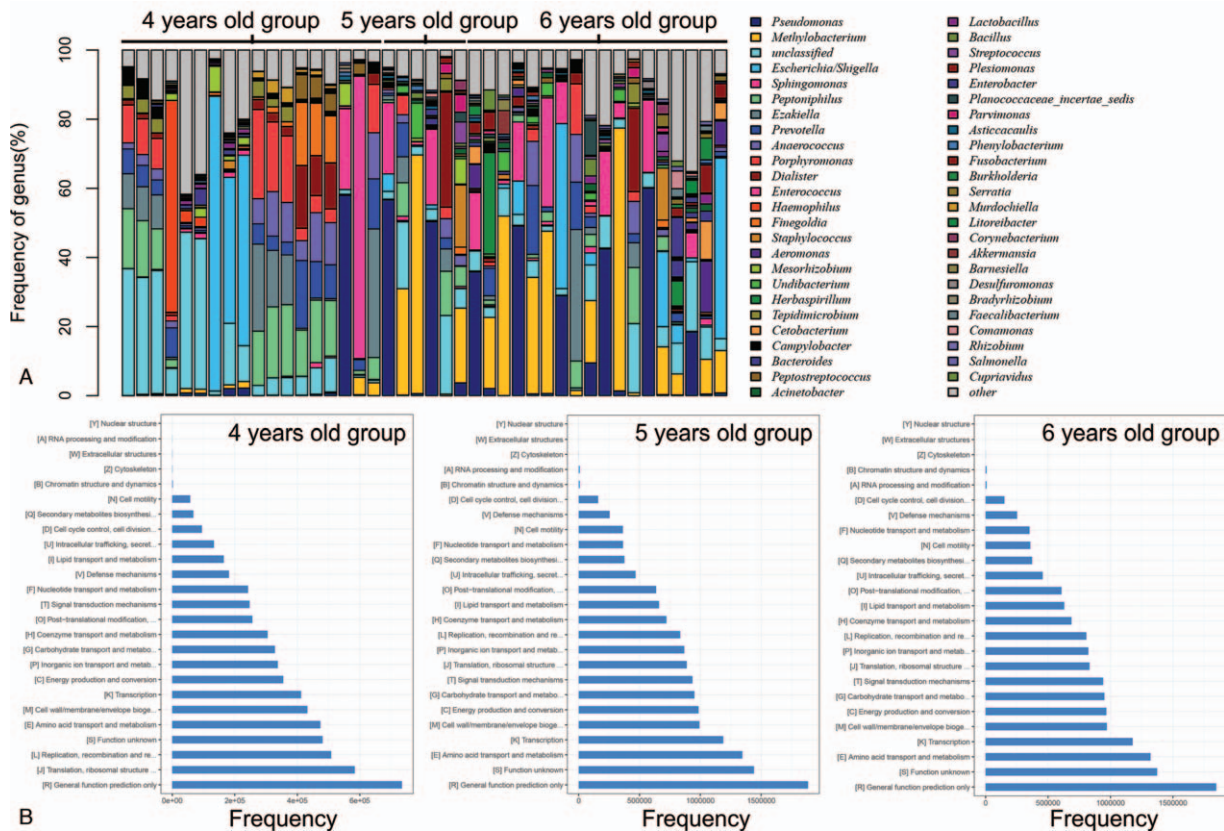
Figure 2. Community structure and bacterial functions of vaginal microorganisms in preschool-aged girls. (A) Distribution map of the community structure of all samples at the genus level. (B) Functional classification of the vaginal discharge of preschool-aged girls based on clusters of orthologous groups. These findings suggest that younger girls exhibit more complex vaginal microecology community structures. Additionally, the bacterial functions changed continuously in the different age groups, and the functions of the vaginal bacteria were more similar and versatile in the 5- and 6-year-old groups.

lism, inorganic ion transport and metabolism, cell membrane biogenesis and signal transduction mechanisms. These findings suggest that the vaginal microecology community structure is more complex in younger children. Additionally, the vaginal bacterial functions were more similar and versatile in the 5- and 6-year-old groups.

4. Discussion

The vaginal flora is a general term for vaginal microorganisms, which are composed of a variety of aerobic bacteria, facultative anaerobes, and anaerobes.[20] An imbalanced vaginal flora can lead to genital infections in women. Untimely diagnosis and treatment can cause serious complications such as pelvic inflammation, premature delivery, and low birth weight and can increase the risks of human immunodeficiency virus infection and cervical cancer.[5,21] Studies have shown that hormonal, behavioral, and physiochemical changes in the genital tract govern the vaginal flora.[22,23] From preschool age to childbearing age, hormone levels in the human body change significantly, thus constituting an important influence on the vaginal flora.[24] In our study, the vaginal flora of preschool-aged girls differed from that of healthy women of childbearing age. The main vaginal flora of preschool-aged girls were Gram-negative bacilli, whereas those of healthy adults were primarily large Gram-positive bacilli such as *Lactobacillus crispatus*. Ravel et al suggested that one or more *Lactobacillus* spp., including

Lactobacillus crispatus, *Lactobacillus iners*, *Lactobacillus gasseri*, and *Lactobacillus jensenii* predominate in the reproductive tracts of most healthy women of childbearing age.[25] Consistent with the results of our study, *Lactobacillus crispatus* and *Lactobacillus iners* are the main vaginal microbes in most Chinese women.[26] Aerobic and anaerobic cultures of the vaginal discharge revealed significantly more bacterial colonies in healthy women of childbearing age than in preschool-aged girls. Additionally, the vaginal discharge of the preschool-aged girls contained significantly more bacterial colonies in the anaerobic cultures than in the aerobic cultures (Fig. 1A). Furthermore, the vaginal microecology diversity was closely related to age in preschool-aged girls (Fig. 1B). Among the 4 to 6-year-old girls, the bacterial diversity decreased with age (Fig. 1C). Additionally, metagenomic sequencing showed that the younger girls had more complex vaginal microecology community structures (Fig. 2A). Furthermore, the bacterial functions changed continuously in the different age groups, and the functions of the vaginal flora were more similar and versatile in the 5- and 6-year-old girls. This was mainly reflected in the functions of energy generation and transformation, amino acid transport and metabolism, inorganic ion transport and metabolism, cell membrane synthesis, and signal transduction mechanisms (Fig. 2B).

Previously, studying the vaginal flora was difficult owing to the limited microbiological research methods. The emergence of culture-based methods has enabled a preliminary understanding of the composition of the vaginal flora.[27,28] However, because

most of the vaginal flora are anaerobic bacteria, which require stricter culture conditions, limitations remain in studying the vaginal microecology in terms of pure bacterial cultures. To date, no culture media fully meet all requirements of all bacteria.[29] Molecular biological methods save the cumbersome steps and complex conditions of culturing, and the microbial community structure can be obtained rapidly and efficiently at the gene-sequencing level, which is no longer limited by culture conditions and the environment. High-throughput sequencing has the advantages of being high-throughput, inexpensive, accurate and fast. 16s rDNA amplifier sequencing yields the entire microbial community information for the vaginal microecosystem at the gene level. This technique is quickly becoming the preferred research method for characterizing the vaginal microbiome composition.[30] 16s rDNA exists in the bacterial genome, is approximately 1550 bp long, and is the most widely used marker gene for bacterial amplicon sequencing. 16s rDNA genes encode the 16s rRNA in prokaryotes and are composed of staggered variable and conservative regions, of which, 9 (V1–V9) are variable and species-specific. In this study, we used the V3 and V4 regions of the variable region, which are the most common variable regions in studying human flora.

This study had some limitations. First, few preschool-aged girls came to the outpatient clinic for vaginal discharge collection, and the DNA concentrations in some samples did not meet the requirements for high-throughput sequencing; thus, limited samples were included in this study. Second, the samples in this study only represent the vaginal flora compositions of preschool-aged girls in the Chengdu area of southwest China, and the environment and subject-specific factors likely affected the results.

In summary, we studied the vaginal flora diversity of healthy preschool-aged girls via sequencing analysis and found that the common dominant vaginal bacteria in preschool-aged girls were *Pseudomonas*, *Sphingomonas*, *Peptoniphilus*, *Prevotella*, and *Aerococcus*, which differed significantly from the dominant vaginal bacteria of healthy women of childbearing age. As the girls' ages increased, the vaginal bacterial composition diversity decreased, and the functional diversity increased. The main colony functions were energy generation and transformation, amino acid transport and metabolism, and signal transduction mechanisms. These findings lay the foundation for a comprehensive understanding of vaginal flora diversity in preschool-aged girls, which can help in clinically diagnosing vulvovaginitis in preschool-aged girls.

Acknowledgments

Author contributions

YDZ and TL drafted the manuscript. TL and JYL participated to acquisition of data. JYL and ZYD generated the experimental results. ZQH and FY designed the study and reviewed the manuscript for intellectual content. All authors approved the final version of the manuscript.
Data curation: Yiduo Zhang, Ting Liu.
Formal analysis: Ting Liu, Jingying Lin.
Project administration: Zhengqiang Hu.
Writing – original draft: Yiduo Zhang.
Writing – review & editing: Fan Yu, Zhengqiang Hu.

References

[1] Overman BA. The vagina as an ecologic system current understanding and clinical applications. J Nurse Midwifery 1993;38:146–51.

[2] Cooperative Group of Infectious Disease CSoO, Gynocology CMA [Expert consensus on the clinical application of vaginal microecology test]. Zhonghua Fu Chan Ke Za Zhi 2016;51:721.

[3] Buck G, Fettweis J, Alves J, et al. The vaginal microbiome: disease, genetics and the environment. Nat Prec 2010.

[4] Champer M, Wong A, Champer J, et al. The role of the vaginal microbiome in gynaecological cancer. BJOG 2018;125:309–15.

[5] Mcclelland RS, Lingappa JR, Srinivasan S, et al. Evaluation of the association between the concentrations of key vaginal bacteria and the increased risk of HIV acquisition in African women from five cohorts: a nested case-control study. Lancet Infect Dis 2018;18:554–64.

[6] Petricevic L, Domig KJ, Nierscher FJ, et al. Characterisation of the vaginal Lactobacillus microbiota associated with preterm delivery. Sci Rep 2014;4:5136.

[7] Wessels JM, Felker AM, Dupont HA, et al. The relationship between sex hormones, the vaginal microbiome and immunity in HIV-1 susceptibility in women. Dis Model Mech 2018;11:dmm035147.

[8] Farage MA, Miller KW, Sobel JD. Dynamics of the vaginal ecosystem—hormonal influences. Infect Dis Res Treat 2010;1–5.

[9] Gerstner GJ, Grtinberger W, Boschitsch E, et al. Vaginal organisms in prepubertal children with and without vulvovaginitis. A vaginoscopic study. Arch Gynecol 1982;231:247–52.

[10] Hickey RJ, Zhou X, Settles ML, et al. Vaginal microbiota of adolescent girls prior to the onset of menarche resemble those of reproductive-age women. Mbio 2015;6: e00097-15.

[11] Cemek F, Odabaş D, Şenel Ü, et al. Personal hygiene and vulvovaginitis in prepubertal children. J Pediatr Adolesc Gynecol 2016;29:223–7.

[12] Jariene K, Drejeriene E, Jaras A, et al. Clinical and microbiological findings of vulvovaginitis in prepubertal girls. J Pediatr Adolesc Gynecol 2019;32:574–8.

[13] Kim H, Chai SM, Ahn EH, et al. Clinical and microbiologic characteristics of vulvovaginitis in Korean prepubertal girls, 2009-2014: a single center experience. Obstet Gynecol Sci 2016;59:130–6.

[14] Joishy M, Ashtekar CS, Jain A, et al. Do we need to treat vulvovaginitis in prepubertal girls? BMJ 2005;330:186–8.

[15] Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. Annu Rev Pathol 2018;14:319–38.

[16] Human Microbiome Project ConsortiumStructure, function and diversity of the healthy human microbiome. Nature 2012;486:207–14.

[17] Simner PJ, Miller S, Carroll KC. Understanding the promises and hurdles of metagenomic next-generation sequencing as a diagnostic tool for infectious diseases. Clin Infect Dis 2018;66:778–88.

[18] Gardner JJ. Comparison of the vaginal flora in sexually abused and nonabused girls. J Pediatr 1992;120:872–7.

[19] Xiao BB, Liu ZH, Liao QP. [Microecological investigation of vaginal microflora in women with varying degree gynecologic symptoms in clinics]. Zhonghua Fu Chan Ke Za Zhi 2009;44:6–8.

[20] Huang B, Fettweis JM, Brooks JP, et al. The changing landscape of the vaginal microbiome. Clin Lab Med 2014;34:747–61.

[21] Davis RW. Diversity of the vaginal microbiome correlates with preterm birth. Reprod Sci 2014;21:32–40.

[22] Brotman RM, Ravel J, Bavoil PM, et al. Microbiome, sex hormones, and immune responses in the reproductive tract: challenges for vaccine development against sexually transmitted infections. Vaccine 2014;32: 1543–52.

[23] Hyman RW, Herndon CN, Jiang H, et al. The dynamics of the vaginal microbiome during infertility therapy with in vitro fertilization-embryo transfer. J Assist Reprod Genet 2012;29:105–15.

[24] Myhre AK, Bevanger LS, Berntzen K, et al. Anogenital bacteriology in non-abused preschool children: a descriptive study of the aerobic genital

flora and the isolation of anogenital Gardnerella vaginalis. Acta Paediatr 2002;91:885–91.

[25] Ravel J, Gajer P, Abdo Z, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci 2011;108(suppl 1):4680–7.

[26] Kedi Wang , Zhi Lv , Jiangrong Su . Research of the vaginal flora in healthy fertile women. J Modern Lab Med 2013;28:1–4.

[27] Zhong W, Millsap K, Bialkowska-Hobrzanska H, et al. Differentiation of lactobacillus species by molecular typing. Appl Environ Microbiol 1998;64:2418–23.

[28] Hillier SL, Krohn MA, Rabe LK, et al. The normal vaginal flora, H2O2-producing lactobacilli, and bacterial vaginosis in pregnant women. Clin Infect Dis 1993;16 suppl 4:S273–81.

[29] Fettweis JM, Serrano MG, Huang B, et al. An emerging mycoplasma associated with trichomoniasis, vaginal infection and disease. PLoS One 2014;9:e110943.

[30] Abayasekara LM, Perera J, Chandrasekharan V, et al. Detection of bacterial pathogens from clinical specimens using conventional microbial culture and 16S metagenomics: a comparative study. BMC Infect Dis 2017;17:631.