WOMEN'S SEXUAL HEALTH

Vaginal Microbiome Is Associated With Vulvodynia, Vulvar Pain Syndrome: A Case-Control Study

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ABSTRACT

Introduction: Vulvodynia, vulvar pain syndrome, is defined as vulvar pain of at least a 3-month duration without a clear identifiable cause, which may have associated factor and the etiology and treatment of this challenging disease is still unclear. Dyspareunia is a relevant symptom of patients with vulvodynia. Vaginal microbiome has known an important role in local immune-inflammatory responses and it may be important pathogenic mechanism in vulvodynia.

Aim: The objective of this study was to investigate the association of vaginal microbiome and vulvodynia.

Methods: We analyzed the microbial compositions of the vestibule and vagina among women with clinically diagnosed vulvodynia (n = 22) and age-matched healthy controls (n = 22) without vulvodynia. The compositions of bacterial microbiomes were compared by pyrosequencing of the 16S rRNA.

Main outcome measure: Vaginal microbiome alpha and beta diversity were assessed using the Shannon diversity index and Heat map. Linear discriminant analysis effect size was used to find out marker for vulvodynia.

Results: There were no significant differences in the age, duration of marriage, history of gynecologic surgery, parity, and menopause status between cases and controls. A total of 1,661,934 high-quality pyrosequencing reads was obtained to evaluate bacterial diversity, and 50,246 unique sequences represented all phylotypes. The type and mean number of the genera were not different between cases and controls. However, the most predominant phyla of bacteria were significantly different between cases and controls. 3 phyla (*Firmicutes, Actinobacteria, and Tenericutes*) and 11 genera including *Gardnerella, Ureaplasma, Achromobacter, Mycoplasma,* and *Bifidobacteria* were significantly more prevalent in cases than in controls ($P < .05$). Linear discriminant analysis effect size analysis suggest the *Bifidobacterium, Mycoplasma, and Fenollaria* species can be potential markers for vulvodynia.

Conclusion: Our results suggest the differences in vaginal microbiome can be associated with the vulvodynia. **Park SY, Lee ES, Lee SR, et al. Vaginal Microbiome Is Associated With Vulvodynia, Vulvar Pain Syndrome: A Case-Control Study. Sex Med 2021;9:100314.**

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INTRODUCTION

Vulvodynia, vulvar pain syndrome, is defined as vulvar pain of at least a 3-month duration without a clear identifiable cause, which may have associated factor and affects up to 8−16% of the female population.[1,2] Vulvodynia is generally regarded as an underdiagnosed, difficult to treat, gynecologic disorder and can be manifested by diverse symptoms such as vulvar burning, stinging, rawness, soreness, or pain.[3,4] Dyspareunia is a relevant symptom of patients with vulvodynia. Vulvodynia negatively affects the quality of partnership and marriage life. The localized (vestibulodynia, clitodynia) or generalized pain (vulvodynia), either provoked or spontaneous, and intermittent or persistent are typically used to classify vulvodynia subtypes. Specific

disorders such as infectious, inflammatory, neoplastic, trauma, iatrogenic, and hormonal deficiencies must be excluded before a diagnosis of vulvodynia is made.[1]

Several theories regarding the underlying etiology have been proposed but still remain unclear.[5] Some studies have proposed that vulvodynia is caused by genital infections, recurrent vulvovaginal candidiasis, herpes simplex virus, human papilloma virus (HPV), contact dermatitis, irritants, or vulvar trauma, but findings have been inconsistent.[6] However, given that the presentations and responses to treatments are relatively diverse, some patients are responsive to medical treatments; however, some patients are refractory to the medical treatments and surgical treatment options including vestibulectomy should be considered;[7] therefore, the optimal vulvodynia care requires a multimodal therapeutic approach[8] and the cause of vulvodynia is most likely multifactorial including microbiota.

Burton et al first reported the association between the vaginal ecosystem and infection in 2003.[9] Previous studies have suggested that breaking of the normal vaginal microbial composition can lead to increased sensitivity to bacterial vaginosis (BV), vulvovaginal candidiasis, and pelvic inflammatory disease (PID).[10,11] Recent studies reported that the composition of the vaginal microbiota also affects infertility, in vitro fertilization outcomes, and pregnancy outcome including preterm labor and delivery.[12–15]

In terms of pain, a recent article suggested that microbial compounds and their receptors can be involved in visceral pain development by activating immune responses.[16] Specific microbiome can induce injury to the vestibular nerve endings, mast cell–induced neuronal hyperplasia via nerve growth factor and heparinase,[17] which may cause severe pain and alterations in the immune response.[18] Autoimmunity may also play a role in the risk of vestibulitis[19] and repeated infection can cause the immune system become more hyperreactive against infected cells. Chronic subclinical yeast infection plays a role in the onset of vestibulitis, although antifungal medications are generally inadequate for patients with undocumented yeast infections.[20] A study also suggested that fibroblast-mediated pro-inflammatory responses to *C. albicans* contribute to the induction of pain in vulvodynia,[21] and the severity of localized provoked vulvodynia is reported to be associated with abnormal vaginal microbiome.[22] Therefore, the association of vaginal microbiome with vulvodynia, painful vulvar disease, needs to be investigated. Metagenomic analysis of the vaginal microbiome by 16S rRNA gene analysis can provide evidence of the significant microbial diversity of the vaginal ecosystem.[10] This study hypothesized that the vaginal and vestibular microbiome of patients with vulvodynia would be different from those of controls without vulvodynia.

MATERIALS AND METHODS

A total of 44 vestibular and vaginal swabs from 22 clinically diagnosed patients with vulvodynia and 22 age-matched healthy controls were collected among women who visited Ewha Womans University Mokdong Hospital between January and October in 2017. The cases were generalized vulvodynia and that the Q-tip test revealed tender sites not confined to the vestibule. The controls were asymptomatic healthy women who visited a clinic for gynecologic screening including pap smear and pelvic sonography without any gynecologic symptoms. Women who were willing to and agreed to participate in this study became the controls and we obtained the written informed consents.

Exclusion criteria are as follows: age below 18 years, pregnancy, diabetes mellitus, use of antibiotics, hormonal contraception or postmenopausal hormone therapy within the previous 3 months, during menstruation, the presence of an intrauterine device, BV, PID, infection with HPV, HIV, chlamydia, Neisseria gonorrhoeae, or Trichomonas vaginalis. All participants completed a careful medical history, a physical examination, wet smear, multiplex PCR and cultures to rule out BV, PID, infection with HPV, chlamydia, Neisseria gonorrhoeae, or Trichomonas vaginalis. 5 times of gentle rubbing on vestibule and vagina with a saline-soaked sterile cotton swab was used to collect a sample. The present study protocol was reviewed and approved by the Institutional Review Board (approval No. EUMC 2017-06-035-002). A written informed consent was submitted by all subjects.

PCR Amplification and Pyrosequencing

PCR amplification of the extracted DNA was performed using primers targeting the V1 to V3 regions of the 16S rRNA gene. For bacterial amplification, barcoded primers of 27F 5″-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-GAG TTTGATCMTGG CTCAG-3″,(the underlined sequence indicates the target region of the primer) and 518R 5″-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-W TTACCGCGGCT G CTGG-3″,(“X” indicates a unique barcode for each subject). DNA was PCR-amplified and PCR products were confirmed by 2% agarose gel electrophoresis. The amplified products were purified. The quality and size of the PCR products were assessed. DNA sequencing was performed by Chunlab, Inc (Seoul, Republic of Korea) using the GS Junior Sequencing system (454 Life Sciences, Branford, CT) in accordance with the manufacturer’s instructions.

Pyrosequencing Data Analysis

The obtained reads of samples were sorted by the unique barcode of each PCR product. The sequences of the barcodes, linkers, and primers were removed from the original sequencing reads. All reads containing 2 or more ambiguous nucleotides, those having low-quality scores (average < 25), and those shorter than 300 bp; in length were discarded. Potential chimeric sequences were detected with the Bellerophon method, by comparisons of the BLASTN search results between forward and reverse half sequences.[23] After removing the chimeric sequences, the taxonomic classification of each read was assigned in

reference to the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net), which contains 16S rRNA gene sequences of type strains that have valid published names and representative species-level phylotypes of cultured and uncultured entries in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) with complete hierarchical taxonomic classification from the phylum to the species level.[24] Sample richness and diversity were determined by Chao1 estimation and the Shannon alpha diversity index at distance of 3%. Random subsampling was conducted to equalize the read sizes of samples to compare various read sizes of the samples. The overall phylogenetic distance between communities was estimated using the Fast UniFrac tool and visualized using principal coordinate analysis.[19] Shared operational taxonomic units were identified by XOR analysis using the CLcommunity program (Chunlab Inc, Seoul, Republic of Korea) and compared among samples.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp. Armonk, NY). The Shannon diversity indices were compared among specimens using the paired t-test and one-way analysis of variance (ANOVA). Pearson's chi-square values and Cohen's kappa indices were calculated to compare the next-generation sequencing data, and the DNA probe assays. A *P*-values <0.05 was considered statistically significant.

RESULTS

Subject Characteristics

Table 1 summarizes the baseline characteristics of each group. Cases and controls were of similar age (mean ± standard deviation [median], 61.0 ± 13.2 [60] years vs 60.6 ± 14.4 [61] years, respectively). Participants were in their thirties to eighties (range, 32-82 years) and age matching was completed before analysis. The median duration of symptoms in the vulvodynia group was 6.1 (range, 3-12) months and severity of pain, as evaluated with the visual analog scale, 0-10 was 8.8 ± 1.3. There were no significant differences in the age, duration of marriage, history of gynecologic surgery, parity, and menopause status between cases and controls.

Sequence Analysis by Pyrosequencing

A total of 1,661,934 high-quality pyrosequencing reads (806,912 sequences from cases and 855,022 from controls) were obtained to evaluate bacterial diversity. Of these, 50,246 unique sequences represented all phylotypes.

Comparison of Phylotype Coverage and Diversity

Table 2 shows analysis of the 2 groups of vaginal communities by a dissimilarity level of 3%, the number of detected operational taxonomic units was close to the total number estimated by the Chao1 and ACE diversity indices. Good's percentage of coverage was ≥99.0% for all sequences. The diversity of the unique species between cases and controls was evaluated using the Shannon index (1.401 vs 1.294, respectively) and the Simpson index (0.457 vs 0.503, respectively) and no significant difference ($P = .28$, parametric ANOVA).

Relative Abundance of Vestibular and Vaginal Microbiota

A total of 48 dominant genera in cases and 42 dominant genera in controls were detected. We listed the most dominant 26 genera in cases and 28 genera in controls in Supplemental Table 1. Lactobacillus was the most often detected in both groups and, when present, was usually the principal taxon. *Streptococcus, Gardnerella, Provotella, Anaerococcus, Corynebacterium*, and *Bacteroides* were the dominant genera in a minority of subjects. An important finding of a heat map of the frequencies of 29 bacterial taxa in cases and controls was that symptoms of vulvodynia occur in women regardless of whether *Lactobacilli* are prominent or not (Figure 1). Bacterial populations were similar between the 2 groups at the genus level. *Gardnerella* and *Lactobacillus helveticus* were more predominant in cases, whereas *Lactobacillus iners* and *Lactobacillus gasseri* were more predominant in controls. Figure 2 shows the overall structure of the vaginal microbiota, which included 7 phyla, regardless of the presence of vulvodynia was revealed. These data showed that

Table 1. Subject characteristics

	Control (n = 22)	Vulvodynia (n = 22)	*P*-value
Age (years)	61.0 ± 13.2	60.6 ± 14.4	.944
Duration of marriage (months)	395 ± 159	432 ± 217	.626
Gynecological operation history (%)	2 (9.1%)	5 (22.7%)	.199
Gravida	3.5 ± 1.6	4.3 ± 2.1	.316
Parity	2.9 ± 1.6	3.0 ± 2.0	.834
Menopause (%)	10 (45.5%)	9 (40.9%)	.674
Duration of symptoms (months)	0	6.1 ± 4.4	NA
Severity of pain (VAS score)	0	8.8 ± 1.3	NA

Data are shown in means± standard deviations.
VAS, visual analog scale 0-10; NA, not available.

Table 2. Comparison of phylotype coverage and diversity estimation of the 16S rRNA gene libraries at the 3% dissimilarity from the pyrosequencing analysis

Group	Reads	OTUs	Good	ACE	95% C.I.
Control	72,149	148.7	99.95	202.8	(−11.25,154.82)
Vulvodynia	65,809	120.62	99.97	131.08	(−11.04,154.60)
Group	Chao1	95% C.I.	Jackknife	Shannon	Simpson
Control	161.23	(−21.31,91.66)	175.25	1.401	0.457
Vulvodynia	126.05	(−20.99,91.34)	136.33	1.294	0.503

The coverage percentage (Good), richness estimators (ACE, Chao1, Jackknife) and diversity indices (Shannon and Simpson) were calculated using Good's method (Good, 1953) and the MOTHUR program.
OTUs, operational taxonomic units; ACE, abundance-based coverage estimator.

most sequences belonged to one of 4 major phyla of *Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria.* Of these, *Firmicutes* and *Actinobacteria* species accounted for the complex vaginal microbiota in cases, whereas *Firmicutes* was the most dominant phyla in controls. The remaining bacteria belonged to the phyla *Cyanobacteria, Fusobacteria*, and *Tenericutes* (approximately 0.1−1.0% of all sequences). At the phylum level, there were significant differences in the composition of *Firmicutes, Actinobacteria*, and *Tenericutes* between cases and controls (one-way ANOVA ($P < .05$)).

Figure 3 shows sequences from the 2 groups comprised 286 different genera, with 283 different genera in cases and 233 in controls. Figure 4 shows significant differences in the proportions of all genera (ie, *Streptococcus, Gardnerella*, *Anaerococcus, Bacteroides, Peptostreptococcus, Atopobium, Moraxella, Ureaplasma, Achromobacter, Mycoplasma,* and *Bifidobacteria*) in cases and controls ($P < .05$). The abundances of *Gardnerella, Ureaplasma, Achromobacter, Mycoplasma*, and *Bifidobacteria* were greater in cases ($P < .05$). The abundances of *Streptococcus, Anaerococcus, Bacteroides, Peptostreptococcus, Atopobium*, and *Moraxella* were greater in controls ($P < .05$).

Linear Discriminant Analysis Effect Size (LEfSe)

The abundance of different species between cases and controls using LEfSe (LDA score >3.5) revealed a total of 7 species were enriched. *Streptococcus, Bacteroides, Anaerococcus*, and *Corynebacterium* species were more enriched in controls than in cases (LDA[log10]scores; 4.32, 4.21, 4.16, and 3.74, respectively, $P < .05$), whereas, there were significantly more *Bifidobacterium, Mycoplasma*, and *Fenollaria* species in cases (LDA[log10] scores; 4.17, 4.09, and 3.87, $P < .01$) (Figure 5).

DISCUSSION

This study demonstrated that relative frequencies of dominant genera, not the diversity of bacterial microbiome, are different between the vulvodynia and the controls, raised the possibility of the vulvodynia can be associated with changes in the proportions of vaginal microbiota, which were mostly apparent at the phylum and genus levels. The most predominant phyla, 3 phyla (*Firmicutes, Actinobacteria, and Tenericutes*) and 11 genera including *Gardnerella*, *Ureaplasma*, *Achromobacter*, *Mycoplasma,* and *Bifidobacteria* were significantly more prevalent in cases than in controls ($P < .05$). The vast majority of vaginal microbiota in controls were *Firmicutes,* whereas *Actinobacteria* and *Tenericutes* were the most prevalent phylum in cases. Lactic acid bacteria (mainly *Lactobacillus*) were the predominant bacterial populations both in the control and vulvodynia groups, as reported in the previous study.[25] Although no studies have yet established the role of *Actinobacteria* and *Tenericutes* in vulvodynia, *Gardnella, Ureaplasma*, and *Mycoplasma* species have been previously shown to be associated with BV.[26] LEfSe analysis suggest the *Bifidobacterium*, *Mycoplasma*, and *Fenollaria* species can be potential markers for vulvodynia. LEfSe is a computational approach for comparisons of biomarker classes to further understand microbial communities and guide biologists to identify novel metagenomic biomarkers.[27]

The diversity of vaginal microbiome was not different between the 2 groups, which is the same result as the previous study reporting the high similarity of the mean number of genera between vulvar vestibulitis syndrome (VVS) and controls.[28] However, a recent study reported a different vaginal microbiome diversity and suggested the possibility of modifying the association between the risk factors and vulvodynia.[29] The genera present in controls were similar to that reported in previous investigations.[9,30] The vaginal microbiome of asymptomatic reproductive aged women showed that *Lactobacillus* species were found in 80.2% and 89.7% in Asian and Caucasian women, respectively.[30] In the present study, *Lactobacillus* species were the most commonly detected and, when present, were usually the predominant taxon, although both groups had generally low dominant rates (59.1% in control and 52.3% in vulvodynia group). This result might have been affected by the greater portion of postmenopausal women (45.5% in controls and 40.9% in cases) in this study because vaginal atrophy in postmenopausal women is negatively correlated with a proportion of *Lactobacillus*. A subanalysis of premenopausal women showed that the proportion of *Lactobacillus* (94.6% in controls and 88.09% in cases) was greater than a previous study reporting 79% in 19 premenopausal Caucasian women.[9] In our results, there was no significant difference in the proportion

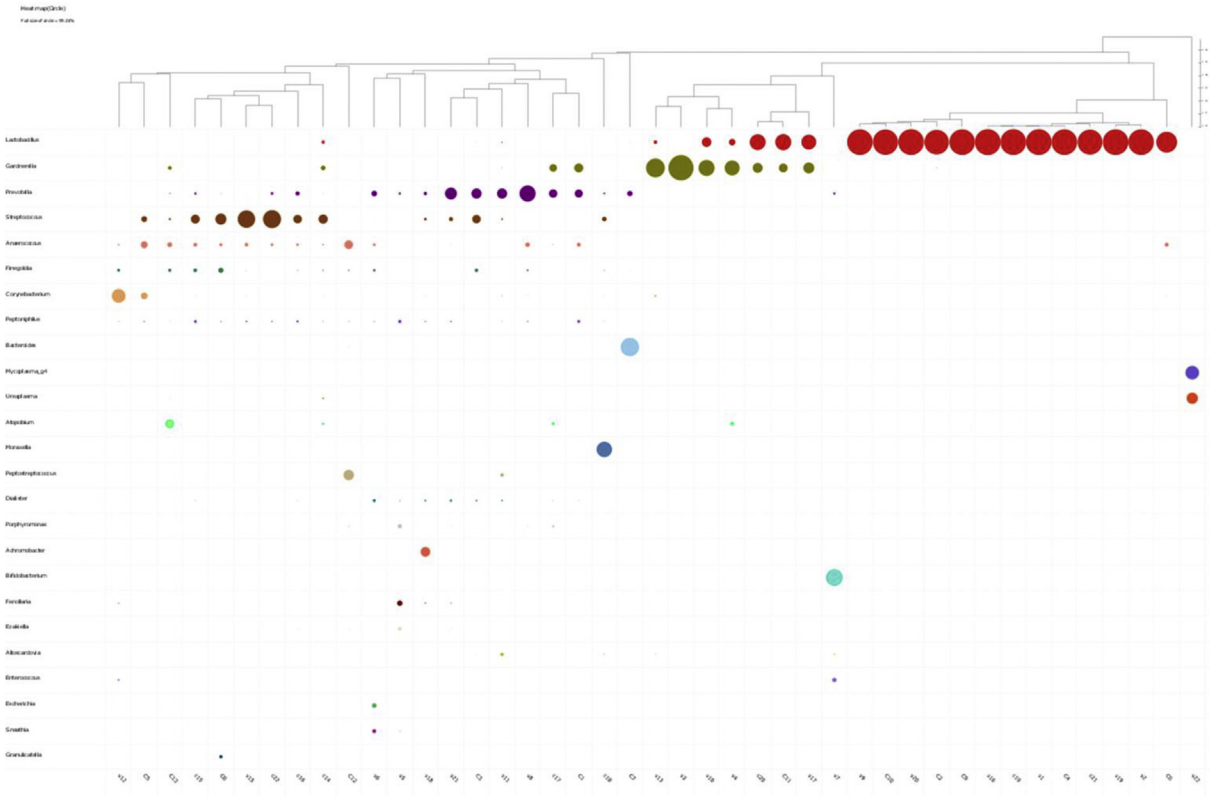

Figure 1. Heat map showing the proportions of 29 bacterial taxa in vestibule and vagina samples from cases of vulvodynia and control subjects. Heat map (circle)-UniFrac clustering. Each column is the sample from one woman and whether they exhibited vulvodynia or controls not is shown in the bottom (C1-22; controls, V1-22; vulvodynia).

of *Lactobacillus* species between the premenopausal and postmenopausal groups. Further studies are needed to elucidate the role of each *Lactobacillus* species. A previous study has reported that the onset of vulvodynia is marked by a decrease in the proportion of *Lactobacillus* species, as well as in the other facultative or anaerobic species, because the vaginal microbial ecosystem changes from eubiosis to dysbiosis.[31] It is highly unlikely that the absence of *Gardnerella* or *Atopobium* species will alter the milieu of vaginal microbes to such an extent as to increase the incidence of vulvodynia symptoms.[28] A pilot study on the microbiome in VVS, a localized form of vulvodynia, found the prevalence of *Streptococcus* and *L. iners* were significantly increased in VVS.[28] Recently, a study concluded that *L. gasseri* either by itself or in combination with other trigger factors was associated with vestibulodynia.[32]

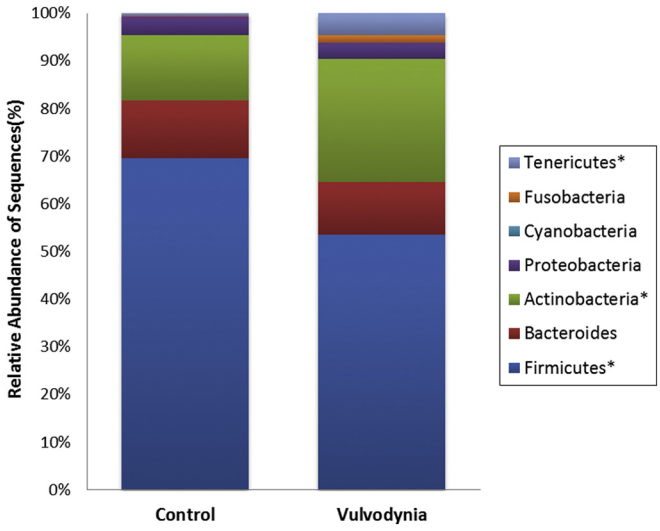

Figure 2. Relative abundance of vestibular and vaginal bacterial V3 tags obtained by pyrosequencing in the vulvodynia and control group, by phylum. *$P < .001$ vs the control group, derived using parametric analysis of variance.

The predominant genera in the vagina suggest a genetic predisposition for the development of vulvodynia by an altered inflammatory response and an increased sensitivity to pain. A study linked genetic polymorphisms to alterations in the function of interleukin (IL)-1β and IL-1 receptor antagonist, and their variant alleles, which have been associated with an increased and prolonged inflammatory response, are found more commonly in women with vulvodynia.[33]

High proportions of *Streptococcus* species are reported to be associated with vaginitis, and metabolic acid is produced by group *B Streptococcus* and may be involved in the onset of tissue toxicity.[34,35] In our study, however, *Streptococcus* species were more dominant in controls than in cases, suggesting that a vaginal flora dominated by streptococci may not be associated with the development of vulvodynia in some women.

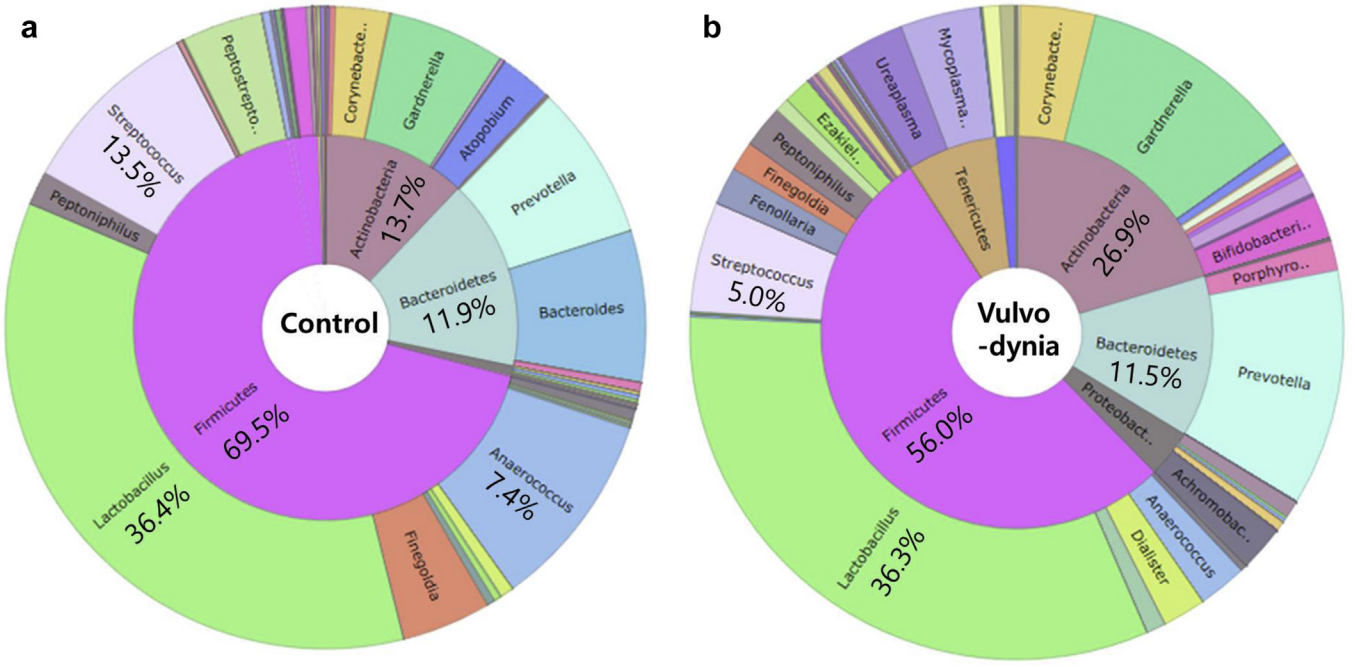

Figure 3. Relative abundance of vestibular and vaginal bacterial V3 tags obtained by pyrosequencing in the (a) control and (b) vulvodynia group, by genus and profiled the overall structure of vaginal communities. Double pie chart; average composition of selected communities (inner circle; phylum, outer circle; genus).

A limitation of our study was the relatively small number of cases. Samples were collected from women with vulvodynia long after the onset of clinical symptoms. Therefore, the possibility that a unique bacterial genus or unique combination of bacteria provided an initial, but transient, trigger for symptoms cannot be eliminated. In addition, more than half of the subjects had low proportions of *Lactobacillus* compared with those in a postmenopausal state. Most of our participants were postmenopausal women (median age of 60 and 61 years old) and this is a limitation of our study considering the recent studies including patients registered in the National Vulvodynia Registry of the United States; 82.2% of women were premenopausal women among 202 participants[36] which were the same in the Belgian and Italian study.[34,37] Therefore, some of the cases of "vulvodynia" in our study potentially could have had pain or tenderness related to genitourinary syndrome of menopause.

Furthermore, the identified genera were the most abundant types present in both groups because the procedures used in this

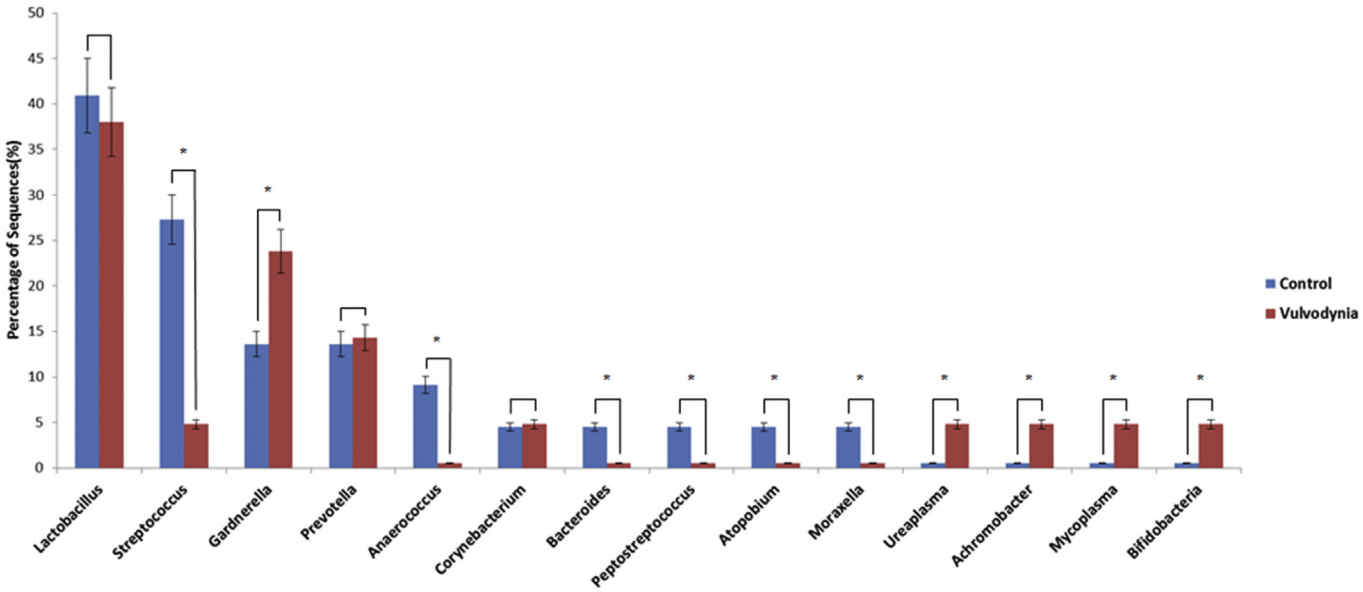

Figure 4. Predominant genera in vestibule and vagina of the vulvodynia and control group. *$P < .05$ vs the control group, derived using parametric analysis of variance.

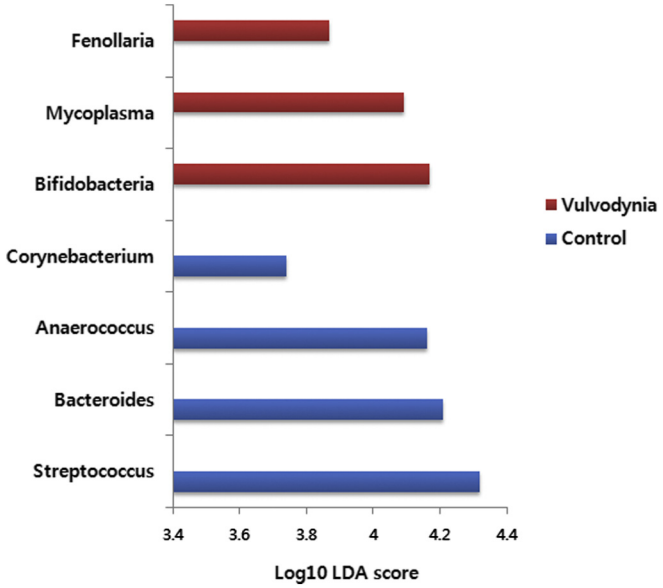

Figure 5. Differentially abundant species between the vulvodynia and control group as determined using linear discriminant analysis effect size (LEfSe) analysis.

study were not applicable for the detection of rare genera. Thus, it is possible that undetected bacterial genera may have contributed to the symptoms of vulvodynia.

However, this study can serve as a starting point for more extensive evaluations as to whether differences in vaginal flora actually influence the symptoms of vulvodynia. Further characterization of vulvodynia subgroups with well-defined etiologies will lead to an improved appreciation of the possible influence of the vaginal microbiota on this syndrome. To the best our knowledge, this study is the first study to describe the vaginal microbiome of Asian women with vulvodynia. These results provide backdrop for future longitudinal studies designed to manage, modulate, and restore homeostasis of and offer an evidence of a causal relationship with vulvodynia to ultimately improve strategies for the treatment and prevention of vulvodynia.

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SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.esxm.2020.100314.