Features of the Vaginal and Vestibular Microbioma in Patients With Vestibulodynia: A Case-Control Study

Filippo Murina, MD,1 Camilla Caimi, MD,1 Francesco Di Pierro, PhD,2 Stefania Di Francesco, MD,1 and Irene Cetin, MD1

1 Lower Genital Tract Disease Unit, V. Buzzi Hospital–University of the Study of Milan, Milan, Italy;

2 Research Department, Velleja Research, Milan, Italy

Corresponding author: Filippo Murina, MD, Via Castelevetro 24, Milan, Italy. E-mail: filippomurina577@gmail.com

ABSTRACT

Objective: Our objective was to determine the role of vaginal and/or vestibular microbiota disturbance as an associated factor of symptom characteristic of provoked vestibulodynia (PVD).

Study Design: In an observational case-control study, the bacterial microbiomes in the vagina and vestibule from 20 women with PVD and 18 healthy controls were compared using a 16S rRNA gene-based molecular analysis. Clinical data were recorded through a 0- to 10-point visual analog scale related to dyspareunia and vulvovaginal pain/burning.

Results: Comparative assessment of the bacterial taxa (cutoff ≥15%) revealed 105 genera in the vaginal samples of PVD patients and 113 genera in the vestibular samples. Similarly, 120 genera were detected in the vaginal samples and 151 in the vestibular samples of the control group. Bacterial complexity was higher in the vestibular samples than in vaginal samples in both groups, without statistically significant differences. The following 3 dominant taxonomic units were found: *Lactobacillus, Gardnerella*, and *Atopobium* in PVD patients and *Lactobacillus, Gardnerella*, and *Bifidobacterium* in the control group. *Lactobacillus gasseri* was dominant only in women with PVD, showing a significant correlation with burning/pain intensity and dyspareunia severity (0.255 and 0.357, respectively, $p < .001$).

Conclusions: Our data suggest that bacterial communities in vaginal discharge are an important contributor to the vestibular microbiota. *Lactobacillus gasseri* may be an element of vulnerability toward the development of vaginal dysbiosis. We can postulate its association as a potential etiologic organism in some individuals, either by itself or in some combination with other trigger factors.

KeyWords:
vaginal microbioma, vestibular microbioma, vestibulodynia, vulvodynia, vulvar pain, lactobacilli

Vulvodynia is defined as a vulvar pain of at least a 3-month duration without a clear identifiable cause, which may have associated factors.1 Provoked vestibulodynia (PVD) is the main subtype of vulvodynia, including approximately 80% of all cases in which the chronic pain is characterized by severe pain with pressure localized to the vulvar vestibule.2,3

No single causative factor of PVD has been identified, suggesting that it is therefore likely of multifactorial origin. It is likely that numerous "triggers" may initiate the pain and thereby a chronic pain condition might develop.

Although various risk factors have been associated with PVD, several lines of evidence point to a pivotal role for inflammation. Several studies reported an increase in the expression of specific proinflammatory cytokines associated with neuroinflammation, including interleukin (IL)-1b and IL-6, in vestibular swabs from women with PVD.4,5 Current understanding suggests that exposure of predisposed women to a variety of exogenous or endogenous triggers may result in vestibular nerve fiber proliferation (peripheral sensitization) and enhanced systemic pain perception (central sensitization).6 Vulvovaginal infections are frequently cited as an inciting inflammatory event triggering the development of PVD. A study suggested that diverse urogenital infections, such as yeast infection, urinary tract infection, trichomonas, and vaginosis, may precede the onset of vulvodynia, with multiple assaults significantly compounding risk.7 Several studies have focused on a proposed link between repeat infections with *Candida albicans* and PVD.8–10 Mimicking repeated vaginal candidiasis that some women with PVD report experiencing, a rodent model of longlasting mechanical allodynia after repeated exposure of the vulva to *C. albicans* has been developed in mice.8

Selective sampling of fibroblasts from painful and adjacent nonpainful sites has demonstrated enhanced proinflammatory cytokine production after yeast extract stimulation.9 Therefore, it is plausible that additional microorganisms may play a role in this inflammatory response, although there is a clear role for *Candida* species.

The aim of this study was to investigate a possible role of vaginal and/or vestibular microbiota disturbance as an associated factor of symptoms characteristic of PVD. We sought to determine whether there are differences in bacterial genera present in paired vaginal and vestibular samples from women with PVD compared with those in samples from control women. In addition, the

purpose of this trial was to investigate the relationship between the vaginal/vestibular microbiome and the PVD.

MATERIALS AND METHODS

Subjects

This case-control study compared women with PVD with control women without vulvar pain. All women aged 18 years and before menopause (absence of menstruation for 12 months) with PVD who presented to our unit of lower genital tract disease were invited to participate.

The control group included asymptomatic healthy women without any vulvovaginal condition who attend our hospital for their routine gynecologic examination. Patients willing to participate provided informed consent and agreed to sign and follow the protocol.

Cases with PVD met the criteria of the International Society for the Study of Vulvovaginal Disease,1 including vulvar pain/ burning/irritation localized to the vestibule during vaginal intercourse and during activities exerting pressure on the vestibule (tampon insertion, tight jeans or pants, cycling, horseback riding) that had been present for at least 3 months, with no other demonstrable cause for their symptoms (e.g., untreated infections, dermatologic disorders).

Exclusion criteria included currently being pregnant or breastfeeding, an active vulvovaginal infection (candidosis, bacterial vaginosis [BV], trichomonas vaginalis, and aerobic vaginitis) at the time of their gynecological examination, genital bleeding of unknown origin, and use of antibiotics or antifungals in the 14 days preceding enrolment.

All enrolled participants completed a detailed questionnaire to assess demographics, including age, marital status, ethnic origin, the number of sexual partners, the use of contraception, and history of medication and vaginal infections. Before any other vaginal examination was performed, a vaginal sample from the upper lateral part of the vagina and another one from the perivestibular area was collected with the help of an unmoistened speculum. Clinical data related to PVD were recorded through a 0- to 10-point visual analog scale (VAS) related to dyspareunia and vulvovaginal pain/ burning (0 = no pain; 10 = worst possible pain).

Avestibular cotton swab test was also collected. It consisted of a small cotton-tipped applicator lightly rolled over the surfaces of the vestibule (mean of values at the 1, 3, 5, 6, 7, 9, and 11 o'clock locations) by asking the subject to report pain intensity through a numeric rating scale in which 0 represents “no pain” and 10 the “worst pain imaginable.” Participants were asked to refrain from vaginal intercourse for 48 hours before sample collection and they presented for enrolment on a day

when no vaginal bleeding was present. The procedures were approved by the institutional review board of V. Buzzi Hospital-University of the Study of Milan and with the Helsinki Declaration.

Bacterial Characterization

For the purpose of this study, vaginal and vestibular swab samples were collected (vestibular sample was collected before the vaginal one) in sterile tubes containing 1 mL of DNA-RNA shield from ZYMO Research until bacterial DNA extraction and shipped to the Department of Chemistry, University of Parma- Italy for analysis. Vaginal and vestibular samples were subjected to DNA extraction using the ZymoBIOMICS DNA miniprep kit following the manufacturer's instructions (ZYMO Research).

Partial 16S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio_Uni and/Probio_Rev, targeting the V3 region of the 16S rRNA gene sequence.11 Illumina adapter overhang nucleotide sequences were added to the partial 16S rRNA gene-specific amplicons, which were further processed using the 16S Metagenomic Sequencing Library Preparation Protocol (Part #15044223 Rev. B – Illumina). To calculate downstream diversity measures (α and β diversity indices, UniFrac analysis), 16S rRNA operational taxonomic units were defined at 100% sequence homology using DADA23; operational taxonomic units not encompassing at least 2 sequences of the same sample were removed. Notably, this approach allows highly distinctive taxonomic classification at single-nucleotide accuracy.12

All reads were classified to the lowest possible taxonomic rank using QIIME2 and a reference data set from the SILVA database. Regarding *Lactobacillus* ITS PCR amplification and sequencing, all reads were classified to the lowest possible taxonomic rank using QIIME24,5 and a reference data set, consisting of an updated version of the *Lactobacillus* ITS database.13

Statistical Analyses

We calculated a sample size of 18 women in each group to have 80% power to detect a 1-log change in microbial densities based on a paired samples t test and a common SD of the microbial density difference of 2.1 observed in a prior study on normal vaginal microbiota.14 To identify possible correlations between taxa and VAS, analysis of covariance and Pearson correlation coefficient were calculated using SPSS software v. 22 (IBM SPSS Statistics for Windows, Version 22.0; IBM Corp, Armonk, NY). T tests were performed to compare the variables.

Role of the Funding Source

The study was supported by the Associazione Italiana Vulvodinia, a nonprofit Italian association whose mission is to improve the health and quality of life of women experiencing vulvodynia and chronic vulvar pain.

RESULTS

Between January 2019 and April 2019, 38 women (20 with PVD and 18 controls) were enrolled and completed the informed consented in this study. Participants ranged in age from 23 to 48 years, and because of matching cases to controls by decade in age, no difference in age was noted between cases (mean, 32.5 ± 8.2 years) and controls (mean, 34 ± 6.2 years; p = NS); all women were of white origin. Furthermore, oral contraceptive use, length of menstrual periods, and body mass index were not statistically significantly different between the study groups. Table 1 presents the anamnestic and clinical characteristics of all women with PVD. The mean ± SD vulvar burning/pain score was 5.8 ± 1.6, the mean ± SD pain during and after intercourse was 7.3 ± 2.4, and the mean number of months since the first vulvar symptoms was 28.5 ± 31.1.

Evaluation of biodiversity for all subsamplings of sequenced read pools revealed (see Figure 1) that vestibular samples had a bacterial complexity higher than that of vaginal samples in both groups, whereas there were no statistically significant differences between patients with PVD and control women. Comparative assessment of the bacterial taxa predicted by 16S analysis maintaining a cutoff of microbiological positivity of 15% or more revealed the presence of 105 genera in vaginal samples in PVD patients, and 113 genera were identified in the vestibule.

Similarly, a total of 120 genera were detected in vaginal samples in the control group, and 151 genera were identified in the vestibule. There was a substantial overlap in the species composition between PVD and control participants, without statistically significant differences in relation to the place of collection (vaginal versus vestibular) in both groups. *Lactobacillus* was most often detected in samples from both sites, and it was the predominant taxon in PVD patients and in the control group. Extrapolating the percentage of dominant genera, defined as any bacterial taxon that constituted more than 25% of a microbiological community, we found the following 3 taxonomic units in both groups: *Lactobacillus, Gardnerella vaginalis*, and *Bifidobacterium* in the control group women and *Lactobacillus, Gardnerella*, and *Atopobium* in the group of women with PVD (Table 2), without a statistically significant difference regarding the site of sampling (vaginal or vestibular).

Although the frequency of *Lactobacillus* dominance in the vagina and vestibule was similar in both groups, interesting differences were noted in the presence of various *Lactobacillus* (L.) species

(Table 3). The vaginas of 70.5% of the controls as opposed to 57.1% of the PVD subjects were dominated by *Lactobacillus crispatus*, whereas *Lactobacillus iners* was the dominant *Lactobacillus* in 35.2% of the controls and 19.0% of PVD subjects. *Lactobacillus gasseri* was not dominant in the control group and was identified as dominant in 19.0 vestibular samples and 14.2 vaginal samples in only the PVD group.

None of the differences between women with PVD and controls reached the level of significance. Analysis of covariance in the PVD group between *Lactobacillus* species and symptoms (burning/pain and dyspareunia) showed a significant correlation of *L. gasseri*, burning/pain intensity, and dyspareunia severity (0.255 and 0.357, respectively, $p < .001$; see Figure 2).

DISCUSSION

The human vaginal microbiota plays a key role in preventing many urogenital diseases, such as BV, yeast infections, and sexually transmitted disease. Whether the microbiota of the vagina and/or vestibule contributes to the initiation and/or perpetuation of pain in women with PVD has not been completely resolved.

The first evidence of our study is that the vaginal and vestibular microbiota composition was similar between women with PVD and controls. More accurately, we found a larger microbial biodiversity of the vestibular ecosystem than the vaginal ecosystem in both groups, without achieving statistical significance, that we can explain as a locoregional contamination at low concentrations.

Our findings support the results of a previous investigation that examined the bacterial population present in paired vaginal and vestibular samples from women with PVD and controls by gene amplification technology, demonstrating a strictly bacterial identity and relative abundance in both sites.15 Although we cannot consider biodiversity as a marker of health or disease related to PVD, our evidence suggests that bacterial communities in vaginal secretions are an important contributor to the vestibule microbiota. *Lactobacillus* dominance was evident in both groups, but *Atopobium vaginae* and *Gardnerella* were identified in the vagina and vestibule at a higher frequency in women with PVD than in controls, whereas *Bifidobacterium* and *Gardnerella* were dominant in the vagina and vestibule of women with PVD.

The advent of molecular approaches based on the cloning and sequencing of 16S rRNA genes has allowed the identification of at least 5 major types of vaginal microbiota, called community state types (CSTs), in reproductive-aged women.16 Four of these CSTs are dominated by *Lactobacillus* and have been associated with healthy reproductive-aged women.17

It was demonstrated that communities in group 1, which occurred in 26.2% of the women sampled, were dominated by *L. crispatus*, whereas groups 2 (6.3%), 3 (34.1%), and 5 (5.3%) were dominated by *L. gasseri*, *L. iners*, and *L. jensenii*, respectively.18

A relevant finding of this study is related to the differences in the presence and frequency of *Lactobacillus* species between the 2 groups. *L. crispatus* was more often dominant than other *Lactobacillus* species in the vagina and vestibule in both study groups, but *L. gasseri* was dominant at both sites in only women with PVD, according to the findings of Ventolini et al 19 who found *L. crispatus* in control patients, whereas *L. gasseri* was detected in patients with symptomatic vulvodynia or vulvodynia in remission.

Similarly, in another trial that compared the bacterial microbiome from women with PVD and controls, *L. gasseri* was not detected in the control group and was identified in 26.7% of women in the PVD group.15 Our findings provide further evidence that observation of *L. gasseri* in PVD is not an accident. *Lactobacillus gasseri* is an indication of a normal vaginal microflora, representing the group 2 CST.20 However, its presence may provide reduced defensive capacity, with a more precarious balance than that in other CSTs, which can easily evolve toward an unstable vaginal microbiota.

In a study conducted among pregnant women, it was demonstrated that a *Lactobacillus*-dominated vaginal microflora containing *L. crispatus* shifted to an abnormal vaginal microbiota in only 2.4% of the cases, whereas a vaginal microbiota containing *L. gasseri* converted to an abnormal *Lactobacillus*-dominated vaginal microflora at a rate of 14.5% of the cases. Accordingly, a normal vaginal microbiota containing *L. gasseri* incurred a tenfold increased risk of conversion to an abnormal *Lactobacillus* dominated vaginal microflora.21 We can also speculate that *L. gasseri* in PVD patients may be an element of vulnerability toward the development of dysbiosis (imbalance of the vaginal microbial community) with colonization of the vagina by anaerobic and microerophilic pathogens. In the PVD group, we found the dominance of *A. vaginae* and *Gardnerella*, typical microorganisms found in women with BV. The ability of individual BV-associated bacterial species to elicit an in vitro inflammatory response has also been studied, as in the cases of *A. vaginae*, which induces expression of IL-6, IL-8, and tumor necrosis factor-α via nuclear factor κB, and *Gardnerella*, which induces IL-6 and IL-8 transcription.22,23

The finding of *Bifidobacterium* dominance in the control group may also be implicated in PVD. Although it is difficult to explain any relevant mechanism, one could hypothesize that *Bifidobacterium* might be beneficial or even protective in the polymicrobial vaginal microbiome of asymptomatic healthy females.24 It is conceivable that the association of *L. crispatus* and *Bifidobacterium* can act as a “protective shield,” even in the presence of *Gardnerella*, which

therefore can assume a harmless role. One of the major strengths of our study is that *L. gasseri* showed a strong significant correlation with burning/pain intensity and dyspareunia severity (0.255 and 0.357, respectively, $p < .001$). This finding concurs at least in part with a report by Ventolini et al,19 in which vaginal samples were obtained from patients with PVD. They demonstrated an increase of almost 35 times for IL-17 ($p = .0001$) and threefold for IL-12 compared with those of matched controls, and *L. gasseri* was found in only patients with symptomatic vulvodynia or vulvodynia in remission.19

Although our study cannot and does not implicate *L. gasseri* in the etiology of PVD, we cannot discount its direct role as a potential etiologic organism in some individuals, either by itself or in some unfavorable combination with other trigger factors (e.g., dysbiosis).

Potential weaknesses of our study include the limited sample size, especially the number of negative controls. However, this series was sufficiently powered to demonstrate a characteristic vaginal and vestibular microbiota in women with PVD, further enhanced by the high correlation between *L. gasseri* with burning/pain intensity and dyspareunia severity.

Avestibular microbiota or microbial product may activate a cascade of inflammatory events leading to increased localized nerve stimulation or sensitivity. The resultant alterations may become permanent or more readily induced by a variety of concurrent and/or subsequent triggers.

Further studies on larger numbers of women with PVD are obviously necessary to validate our suggestions, and we envision that this research will later impact novel therapeutic options targeted to resolve early microbial colonization or alterations in the vaginal and vestibular microbiome in women with PVD.

The authors have declared they have no conflicts of interest.

The institutional review board approval was obtained on December 10, 2018, by an ethical committee of V. Buzzi Hospital-University of the Study of Milan (Project Number #34587/2018).

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TABLE 1. Distribution of Symptoms and Signs in Patients With PVD

Parameter	Values
Duration of symptoms, mo	28.5 ± 31.1
History of infections°	11/20 (55%)
Burning/pain (VAS)	5.8 ± 1.6
Dyspareunia (VAS)	7.3 ± 2.4
Cotton swab test*	6.2 ± 1.9

Data are presented as mean ± SD or n (%).

*Score from 0 = no pain to 10 = worst possible pain.

TABLE 2. Dominant Bacterial Genera in the Vagina and Vestibule of Patients With Vestibulodynia and Healthy Women (Control Group)

	Vestibulodynia		Controls	
Taxonomic unit	**% positive**	**% dominant**	**% positive**	**% dominant**
Vagina				
Lactobacillus	100.0	90.4	94.1	64.7
Gardnerella	28.5	4.7	64.7	17.6
Atopobium	23.8	9.5	0.0	0.0
Bifidobacterium	18.2	0.0	70.5	5.8
Vestibule				
Lactobacillus	100.0	80.9	100.0	64.7
Gardnerella	61.9	9.5	76.4	11.7
Atopobium	47.6	9.5	25.2	0.0
Bifidobacterium	21.2	0.0	82.3	5.8

TABLE 3. Lactobacillus Species in the Vagina and Vestibule From Patients With Vestibulodynia and Control Women

	Vestibulodynia		Controls	
Species	**% positive**	**% dominant**	**% positive**	**% dominant**
Vagina				
L. crispatus	85.7	57.1	94.1	70.5
L. iners	52.3	19.0	76.4	35.2
L. jensenii	28.5	9.5	35.2	0.0
L. paracasei	100.0	0.0	94.1	0.0
L. delbrueckii	14.2	0.0	29.4	0.0
L. fermentum	14.2	0.0	17.6	0.0
L. gasseri	38.10	14.2	23.5	0.0
L. reuteri	14.2	0.0	35.2	0.0
L. sakei	0.0	0.0	23.5	0.0
L. plantarum	0.0	0.0	29.4	0.0
Vestibule				
L. crispatus	90.4	61.9	76.47	64.71
L. iners	76.1	23.8	82.35	52.94
L. jensenii	28.5	0.0	41.18	5.88
L. paracasei	90.4	0.0	88.24	0.0
L. delbrueckii	23.8	0.0	23.53	0.0
L. fermentum	0.0	0.0	23.53	0.0
L. gasseri	57.1	19.0	23.53	0.0
L. reuteri	23.8	0.0	17.65	0.0
L. sakei	0.0	0.0	0.0	0.0
L. plantarum	0.0	0.0	35.2	0.0

Dominant genera = bacterial taxon that constituted >25% of a microbiological community.

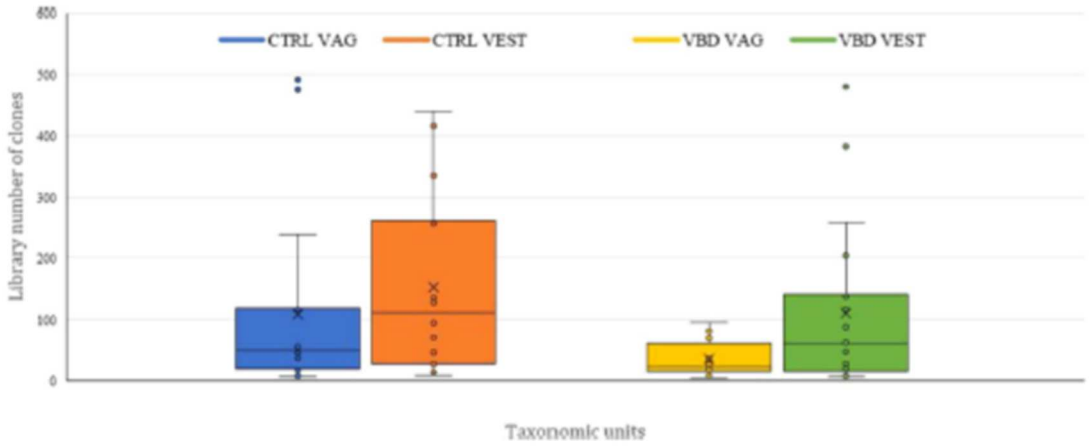

FIGURE 1. Rarefaction curves of vaginal and vestibular microbiological biodiversity. CTRL indicates control group; VAG, vaginal sample; VBD, vestibulodynia group; VEST, vestibular sample.

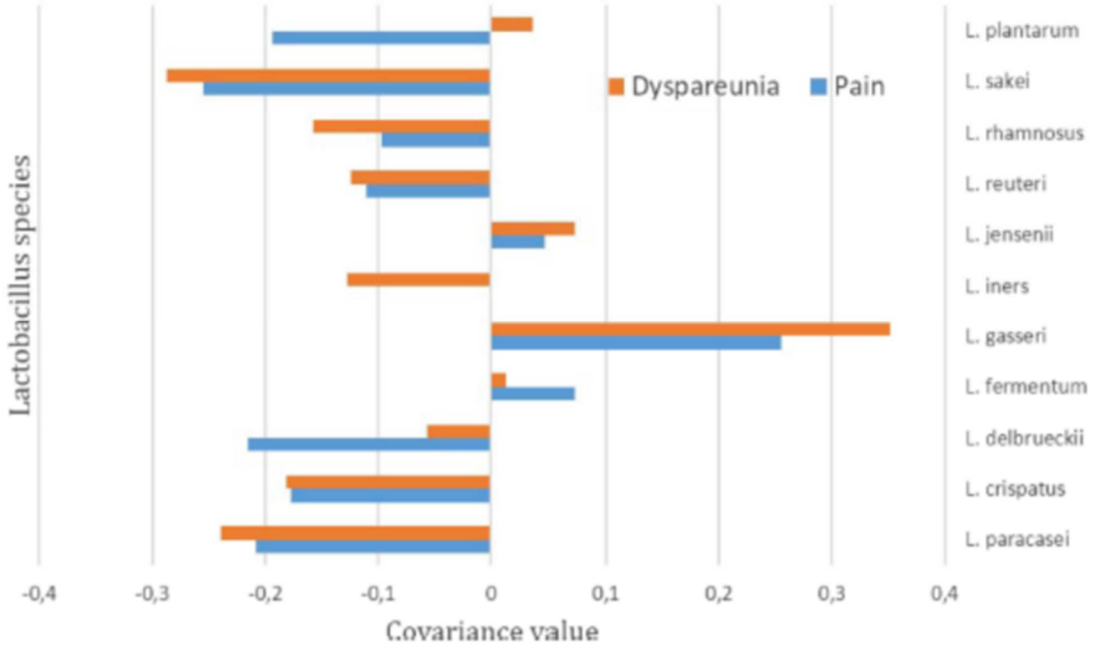

FIGURE 2. Analysis of covariance in the vestibulodynia group between *Lactobacillus* species and symptoms.