GYNECOLOGY

Characteristics of the vaginal microbiome in women with and without clinically confirmed vulvodynia

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BACKGROUND: Vulvodynia (idiopathic vulvar pain) affects up to 8% of women by age 40 years, has a poorly understood etiology, and has variable treatment efficacy. Several risk factors are associated with vulvodynia from a history of yeast infections to depression and allergies. Recent work suggests an altered immune inflammatory mechanism plays a role in vulvodynia pathophysiology. Because the vaginal microbiome plays an important role in local immune-inflammatory responses, we evaluated the vaginal microbiome among women with vulvodynia compared with controls as 1 component of the immune system.

OBJECTIVE: The objective of the study was to characterize the vaginal microbiome in women with clinically confirmed vulvodynia and age-matched controls and assess its overall association with vulvodynia and how it may serve to modify other factors that are associated with vulvodynia as well.

STUDY DESIGN: We conducted a case-control study of 234 Minneapolis/Saint Paul—area women with clinically confirmed vulvodynia and 234 age-matched controls clinically confirmed with no history of vulvar pain. All participants provided vulvovaginal swab samples for culture-based and non-culture (sequencing)—based microbiological assessments, background and medical history questionnaires on demographic characteristics, sexual and reproductive history, and history of psychosocial factors. Vaginal microbiome diversity was assessed using the Shannon alpha diversity Index. Data were analyzed using logistic regression.

RESULTS: Culture and molecular-based analyses of the vaginal microbiome showed few differences between cases and controls. However, among women with alpha diversity below the median (low), there was a strong association between increasing numbers of yeast infections and vulvodynia onset, relative to comparable time periods among controls (age-adjusted odds ratio, 8.1, 95% confidence interval, 2.9—22.7 in those with 5 or more yeast infections). Also among women with low-diversity microbiomes, we observed a strong association between moderate to severe childhood abuse, antecedent anxiety, depression, and high levels of rumination and vulvodynia with odds ratios from 1.83 to 2.81. These associations were not observed in women with high-diversity microbiomes.

CONCLUSION: Although there were no overall differences in microbiome profiles between cases and controls, vaginal microbiome diversity influenced associations between environmental and psychosocial risk factors and vulvodynia. However, it is unclear whether vaginal diversity modifies the association between the risk factors and vulvodynia or is altered as a consequence of the associations.

Key words: case-control studies, microbiome, vulvodynia, yeast infections

Vulvodynia is defined as idiopathic vulvar pain of at least 3 months' duration and is estimated to affect up to 8% of women during their reproductive years.[1,2] Vulvodynia is distinct from vulvar pain related to inflammation, neoplasm, or traumatic injury.[3] Proposed risk factors can be grouped broadly into pathophysiologic (eg, hormonal, inflammatory, environmental) and psychosocial factors (eg, mood, childhood victimization, and other psychosocial events), and etiology likely involves a combination of biopsychosocial exposures that impact a heterogeneous patient population.[3]

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Previous research suggests an altered immune-inflammatory response may be an important pathogenic mechanism.[4] Both clinical- and laboratory-based studies have found associations between vulvodynia and increased production of the proinflammatory cytokine interleukin-1β.[5,6] Further research by Harlow et al[7] found that women with vulvodynia were 2.5 times more likely to self-report hives prior to first report of vulvar pain relative to a comparable exposure time period among controls (95% confidence interval, 1.7—4.4). Furthermore, women with vulvodynia were 5.5 (1.7—17.8) times more likely to report 10 or more yeast infections during their lifetime before vulvodynia onset relative to controls during a comparable time period after adjusting for age, age at first sexual intercourse, and antecedent urinary tract infections.[8]

The vaginal microbiome plays an important role in local immune-inflammatory responses.[9] A more diverse vaginal microbiome is associated with increased inflammatory cytokine levels and Lactobacillus-dominated microbiomes with decreased proinflammatory cytokine levels.[10]

A recent study of 30 women with vulvar vestibulitis syndrome and 15 controls showed no statistically significant vaginal microbiome differences,[11] but there were varying rates of colonization with candida, lactobacillus species, and streptococcus. A second study assessed only culture-based differences between 50 cases of vulvodynia and 50 clinic-based controls and reported a reduction in the diversity of Lactobacilli and greater prevalence of candida and other fungi among cases compared with controls.[12] Our substantially larger study expands on previous work, using a community-based sample of clinically confirmed cases of vulvodynia and controls matched on cases' age at diagnosis of vulvodynia.

AJOG at a Glance

Why was this study conducted?
Vulvodynia affects 3−7% of reproductive-aged women and its etiology is relatively undefined.

Key findings
Characteristics of the vaginal microbiome do not differ between women with and without vulvodynia. However, known associations between certain risk factors and vulvodynia differ by the diversity of the vaginal microbiome.

What does this add to what is known?
Although our study supports a previous finding that characteristics of the vaginal microbiome are not associated with vulvodynia, our novel results suggest that the diversity of the vaginal microbiome may promote or reflect an environment that enables other factors to influence the risk of vulvodynia.

Materials and Methods

Study design/study population
We analyzed data and samples from a case-control study of vulvodynia among women 18−40 years old described previously.[1,8] Briefly, ~30,000 women completing a screener survey at 1 of approximately 40 community health clinics in the Minneapolis/Saint Paul metropolitan area between March 2010 and October 2013 were eligible.

Those likely to meet the International Society for the Study of Vulvovaginal Diseases criteria for vulvodynia and randomly selected controls with no history of vulvar discomfort were invited to participate in a clinical visit. Of the approximately 1400 women invited for further vulvar pain assessment, 350 completed their examination and 234 were clinically confirmed as cases of vulvodynia.

Of the 2287 control women invited, 251 agreed to participate and 234 were clinically confirmed as having no ongoing or past history of vulvar pain. Controls who were the same age or older as a case's age at first onset of vulvar pain were randomly matched to cases and assigned a reference age identical to the age at first onset of vulvar pain in the matched case. For example, a 30 year old woman with vulvodynia onset at age 25 years was matched to a woman with no history of vulvodynia who was at least 25 years of age. Then 25 was considered the reference age and certain exposures of interest were obtained prior to that reference age for both cases and controls.

Data collection: outcome and exposure assessments
All participants completed the clinical visit that followed a standardized approach to confirm the presence or absence of vulvodynia, including a careful medical history, a physical examination with pH assessment, wet preparation, and cultures to rule out vaginitis, especially candidiasis, dermatosis, irritants, or allergens.

In keeping with the diagnostic criteria set by the International Society for the Study of Vulvovaginal Diseases and the most recent National Institutes of Health consensus conference on vulvodynia, the diagnosis of vulvodynia was made by the following: a history of vulvovaginal pain, spontaneous or elicited by touch, and skin findings that are limited to erythema of the vulva or vagina.[13] Participants could not have an active yeast infection defined by symptoms of burning, pruritis, or vulvar irritation accompanied by documented yeast in the vaginal fluid by microscopy or culture at the time of study enrollment. Cases and controls completed several structured questionnaires to provide detailed information about their reproductive and medical history, personal hygiene, and psychosocial factors that preceded the onset of their vulvar pain or comparable age period among controls.

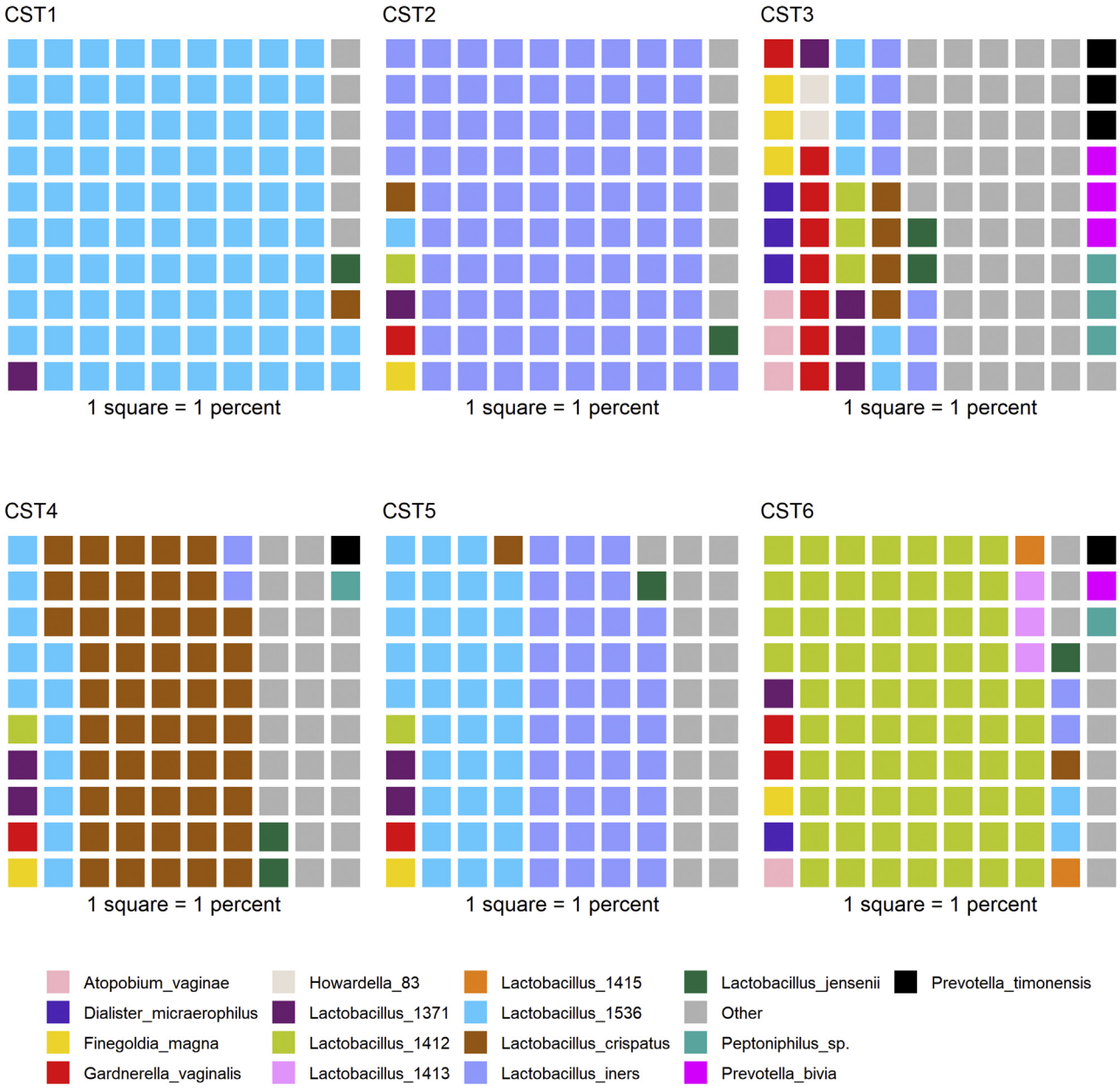
Microbial collection and laboratory methods
A vulvar specialist used 3 Dacron cotton swabs to collect vaginal fluid samples by inserting the swabs simultaneously into the introitus about 1 inch and then rotating 5 times. The first 2 swabs were used for quantitative vaginal cultures and were inserted into a Port-A-Cul transport tube and then capped and shipped to the Sharon Hillier Laboratory at Magee-Womens Research Institute. The third swab was inserted into a cryovial, labeled, and then stored at −70°C. No media or buffer was used in this tube. This specimen was shipped to the Betsy Foxman laboratory and used for 16S ribosomal RNA (rRNA) sequencing.

A detailed description of the methods for obtaining microbial counts is described in the supplementary methods (Supplemental Appendix). In brief, classical culture-based methods were used to quantitate the number of bacteria in the vaginal swab using selective media and standard counting and classification methods (eg, Nugent score).

16S rRNA sequencing and bioinformatics
DNA extraction was performed using Mag Attract power microbiome kit (Qiagen, catalog number 27500-4-EP, QIAGEN Inc, Germantown, Maryland) with glass bead plates. The 16S rRNA V4 region was amplified and sequenced using the dual indexing sequencing protocol.[14] Analysis of the 16S rRNA V4 hypervariable region began with a modified MOTHUR SOP, originally developed by Kozich et al.[14] During this process, sequences were paired and aligned to the SILVA v.123 reference database. Sequencing was performed on the Illumina MiSeq platform using Miseq reagent kit V2 at 500 cycles (Illumina, catalog number MS102-2003).

In lieu of preclustering and clustering via the Mothur standard operating procedures, oligotyping was conducted utilizing an unsupervised minimum entropy decomposition method.[15,16] Species-level classification was assigned using the open source dada2 version 1.6.0 R package.[17] For oligotypes that remained unclassified, representative sequences that uniquely

FIGURE 1
Composition of community state types resulting from clustering of microbiomes across participants using Dirichlet multinomial mixed models



Each *square* represents 1% of the average relative abundance of the samples assigned to that community state type. Taxa whose average relative abundance across all communities state types was less than 2% are grouped into the category of other. Lactobacillus taxa identified as different oligotypes that could not be assigned to a specific species are noted by number (eg, Lactobacillus 1536 shown in *turquoise*).

CST, community state type.

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matched an NCBI BLAST+ species-level taxonomic suggestion at greater than or equal to 98% identity were assigned. If taxa could not be assigned, the oligotype is noted by genus and oligotype number.

There were 13,732,126 reads used in the analysis. The range per person was 1520−87,493, with a median of 24,613. To reduce the complexity of the count data, we calculated alpha diversity using the Shannon diversity index and used the median alpha diversity to classify cases and controls into high (median or greater) and low (less than median) diversity groups. Shannon diversity takes into

TABLE 1
Demographic characteristics of 215 women with chronic vulvar pain (cases) and 222 women with no vulvar pain history (controls), 2010–2015

	Cases	Controls
	n (%)	n (%)
Characteristics	215 (49.2)	222 (50.8)
White race	200 (93.02)	206 (92.79)
Age, y		
18–25	53 (24.6)	29 (13.1)
26–30	78 (36.2)	65 (29.3)
31–35	61 (28.3)	66 (29.7)
36–40	23 (10.7)	62 (27.9)
BMI		
<20	28 (13.0)	20 (9.0)
20–25.9	104 (48.3)	110 (49.6)
26–30.9	48 (22.3)	44 (19.8)
≥31	35 (16.2)	48 (21.6)
Sexual and reproductive history		
Sexual partners (lifetime)		
<5	99 (46.0)	96 (43.2)
5–10	58 (26.9)	46 (20.7)
≥10	58 (26.9)	80 (36.0)
Years on hormonal contraceptive		
<5	78 (36.2)	70 (31.5)
5–10	67 (31.1)	61 (27.5)
≥10	70 (32.5)	91 (41.0)
Ever pregnant	103 (47.9)	130 (58.6)
Nulliparous	129 (60.0)	107 (48.2)
Gynecological infections[a]		
0	115 (53.4)	145 (65.3)
1	66 (30.7)	55 (24.8)
>2	34 (15.8)	22 (9.9)
History of BV	80 (37.2)	54 (24.3)
History of UTI	150 (69.7)	117 (52.7)
Antecedent yeast infections		
0	118 (54.8)	141 (63.5)
1-4	62 (28.8)	61 (27.5)
≥5	35 (16.2)	19 (8.6)
Postyeast infections		
0	58 (26.9)	93 (41.9)
1-4	76 (35.3)	90 (40.5)
≥5	81 (37.6)	39 (17.6)

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account the relative abundance (that is, the evenness of taxa in the community) and the number of taxa (richness). To assess whether associations with alpha diversity were attributable to differences in evenness and/or richness, we categorized cases and controls into high (median or greater) and low (less than median) groups by evenness and richness.

To identify community state types (CSTs), we used an unsupervised clustering method appropriate for compositional data, Dirichlet multinomial mixture models[18-20]; R version 3.4.0 with Dirichlet Multinomial version 1.20.0 package). The number of CSTs was determined by comparing the Laplace approximation of the negative log models and identifying the point at which an increase in Dirichlet components resulted in minor reductions of model fit.

As shown in the waffle plot (Figure 1), each square represents a 1% contribution to the average relative abundance across samples assigned to that community state type. Any species that has an average relative abundance of less than 2% in all 6 community state types is grouped into the category of other for this figure.

We have also provided a heat map (Supplemental Figure 1) in which the colors represent square root–transformed counts, with darker colors representing larger counts. The thicker bands (big blocks of color) are averages across all the samples in that community state type, and these can be used to determine the dominant species in that CST. Further details can be found in the Supplemental Appendix.

Lastly, we used ALDEx2 version 1.20.0[16,17] to investigate associations of individual taxa abundance by vulvodynia status and community state type. This package transforms taxon abundances for each sample to centered-log ratios and calculates a median centered log ratio for each group of interest. The effect sizes for each taxon calculated by ALDEx2 summarize the ratio of between-group differences to within-group differences. Statistical significance for effect sizes were calculated using the Wilcoxon rank-sum test with a false discovery rate–corrected *P* values us-

TABLE 1
Demographic characteristics of 215 women with chronic vulvar pain (cases) and 222 women with no vulvar pain history (controls), 2010–2015 *(continued)*

	Cases	Controls
	n (%)	n (%)
Characteristics	215 (49.2)	222 (50.8)
Allergenic exposures and relevant conditions and medications		
Reported antibiotic use (>6 months)	19 (8.8)	13 (5.9)
Any allergy[b]	195 (90.7)	181 (81.5)
History of hives	86 (40.0)	67 (30.2)
History of insect stings[c]	14 (6.5)	8 (3.6)
Seasonal allergies	125 (58.1)	111 (50.0)
History of autoimmune disease[d]	24 (11.1)	15 (6.8)
Positive history of functional somatic syndromes[e]	103 (47.9)	44 (19.8)
Antecedent functional somatic syndromes	61 (28.3)	21 (9.5)
Psychosocial factors		
History of mod/severe abuse	125 (58.1)	114 (51.4)
Antecedent anxiety	68 (31.6)	44 (19.8)
Post anxiety	39 (18.1)	31 (14.0)
Antecedent mood	66 (30.7)	50 (22.5)
Post mood	49 (22.7)	50 (22.5)
Rumination (tertiles)		
T1	56 (26.0)	82 (36.9)
T2	63 (29.3)	71 (32.0)
T3	79 (36.7)	59 (26.6)
Microbiome characteristics		
Community state type		
CST1-2	113 (52.6)	108 (48.7)
CST 3	34 (15.8)	44 (19.8)
CST 4-6	68 (31.6)	70 (31.5)

BMI, body mass index; *BV*, bacterial vaginosis; *CST*, community state type; *UTI*, urinary tract infection.

[a] Gynecological infections include the total number of gynecological infections (gonorrhea, genital warts, bacterial vaginosis, trichomoniasis, pelvic inflammatory disease, chlamydia, genital herpes); [b] Any allergy includes seasonal, sinus infections, contact dermatitis, medications, and food; [c] History of stings includes more than 1 sting with moderate and severe reaction; [d] Autoimmune conditions include Sjogren's syndrome, Crohn's disease or ulcerative colitis, diabetes, multiple sclerosis, alopecia areata, celiac disease, scleroderma, systemic lupus erythematosus, and rheumatoid arthritis; [e] Functional somatic syndromes include interstitial cystitis, chronic fatigue syndrome, temporomandibular joint disorder, irritable bowel syndrome, and fibromyalgia.

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ing the Benjamini-Hochberg correction. The parameters used for this step included 1000 Monte-Carlo simulations.

Statistical analyses

Complete microbiome sequencing data were available for 215 cases and 222 controls. For this analysis, the reference age matching was not retained but assessed as a covariate, particularly because we assessed the impact of the vaginal microbiome as an effect measure modifier.[21] We first described demographic, medical, and psychosocial characteristics between those with and without vulvodynia and the distribution of factors associated with microbial features (for example antibiotic use, autoimmune conditions, history of bacterial vaginosis). We then fit logistic regression models to estimate the odds ratios and 95% confidence intervals for selected risk factors and vulvodynia adjusted for age and stratified by Shannon alpha diversity above the median (high) and below the median (low).

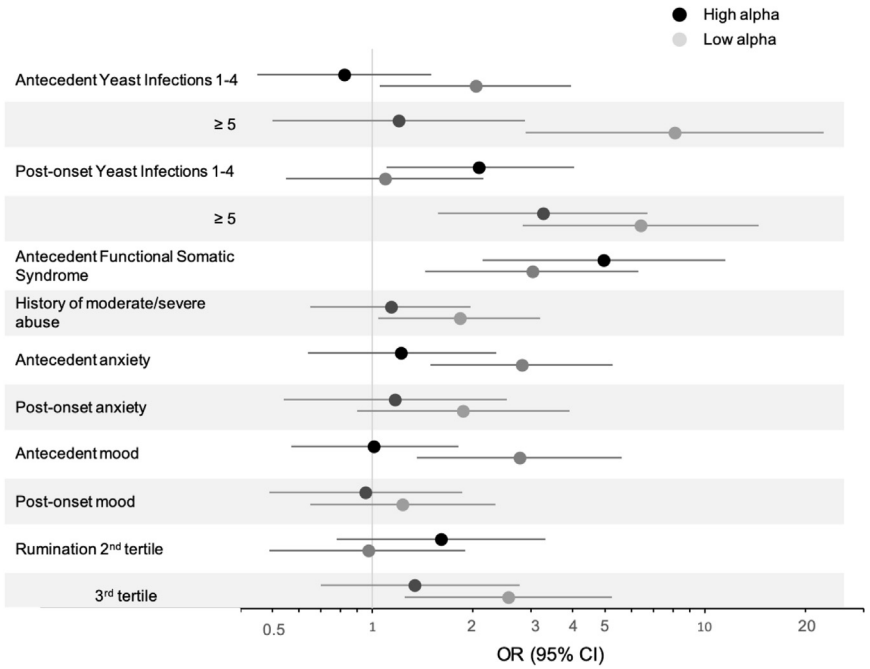
Similar models were fit stratified by categorical CSTs. CSTs were determined using Dirichlet multinominal mixed models, and unsupervised clustering method described in the Supplemental Appendix. CST1, CST2, and CST3 were treated as separate groups, and CST4–6 were combined because of small numbers and similar patterns of diversity (eg, multiple organism dominant). The analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC).

Results

Vulvodynia cases were, on average, 2 years younger than controls but were similar in body mass index (Table 1). Cases reported fewer sexual partners, pregnancies, and years of contraceptive use than controls. As previously shown,[8] cases were more likely than controls to report a history of gynecological infections, allergenic exposures (eg, seasonal allergies, hives, and insect bite sensitivities), autoimmune diseases, and a history of other comorbid functional somatic syndromes. Cases were also more likely than controls to report a history of psychosocial and psychological morbidity including a history of childhood abuse, anxiety, depression, and rumination preceding their first onset of vulvar pain.

Culture-based analyses of the vaginal microbiome showed no meaningful differences between cases and controls (see Supplemental Table 1). At the time of specimen collection, cases and controls had similar numbers of neutrophils and Nugent scores in vaginal smears. Quantitative vaginal culture results showed no differences between cases and controls for H_2O_2 negative or positive

FIGURE 2
Age-adjusted odds ratios for yeast infections and psychosocial factors stratified by alpha diversity



Vulvodynia cases (n = 215) and matched controls (n = 222), 2010–2015.

CI, confidence interval; *OR*, odds ratio.

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Lactobacillus species, *Gardnerella vaginalis*, *Enterococcus*, *Escherichia coli*, viridans group or Group B streptococci, and a number of anaerobic organisms. No differences were seen by culture-based methods when stratified by Shannon diversity. All confidence intervals shown in Supplemental Table 1 include 1.0.

The vaginal microbiome Shannon alpha diversity was similar between cases and controls (see Supplemental Figure 2) as was the distribution of community state types, shown in Table 1. However, we observed effect measure modification of associations for several risk factors for vulvodynia relative to comparable age periods among controls, by whether women had a vaginal microbiome with Shannon alpha diversity below the median (low) or above the median (high) (Figure 2).

Among those with low diversity but not among those with high diversity, there was a strong association between vulvodynia and increasing numbers of yeast infections prior to vulvodynia onset, history of moderate to severe childhood abuse, antecedent (prior to vulvar pain onset or comparable time period among controls) anxiety, depression, and high levels of rumination with odds ratios ranging from 1.83 to 2.81 (Figure 2). The association with yeast infections remained strong after adjustment for psychosocial risk factors (Supplemental Table 2).

We repeated this analysis by measures of evenness and richness. Similar to the effect modification observed by alpha diversity with the associations with antecedent yeast infections, there was also effect modification by evenness. This was not true for richness (Supplemental Figures 3 and 4). This suggests that the associations with alpha diversity were mainly attributable to uneven distributions of taxa.

The associations between psychological factors and vulvodynia among women with low alpha diversity microbiomes remained strong after adjustment for antecedent yeast infections and history of urinary tract infections (Supplemental Table 3). Of note, a history of other comorbid pain conditions was strongly associated with vulvodynia irrespective of alpha diversity.

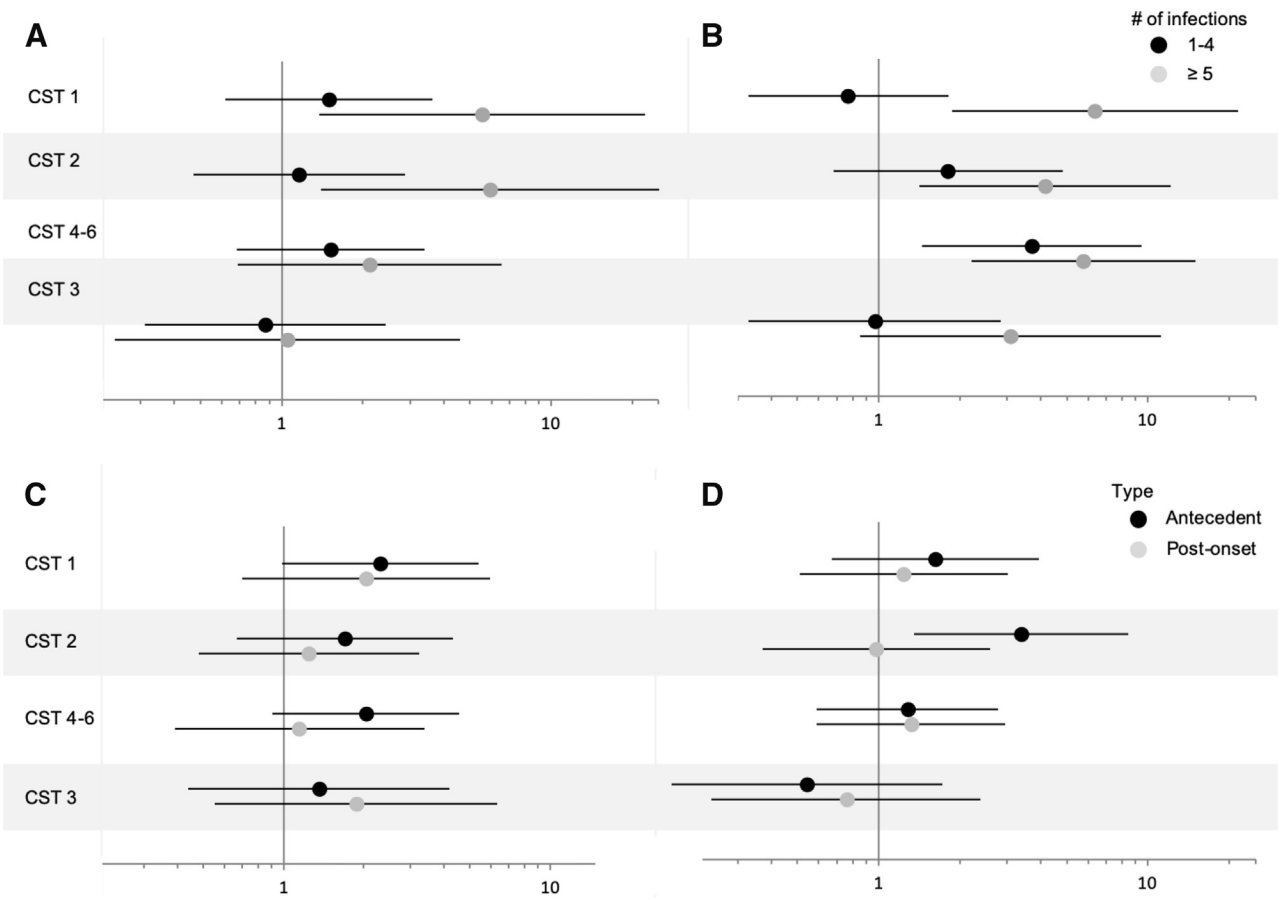
Also, the risk of yeast infections subsequent to vulvodynia onset and risk of psychiatric conditions subsequent to vulvodynia onset did not vary by alpha diversity in comparison with these risk factors prior to the onset of vulvar pain (or comparable time period among controls). We observed no effect measure modification by alpha diversity when we assessed the associations between a history of allergies, medications, and medical conditions and vulvodynia.

We next stratified analyses by microbiome CST membership assigned using Dirichlet multinomial mixed models, shown in a waffle plot (Figure 1). For these CST analyses, CST groups 4,5, and 6 were combined into 1 group because of low numbers and because they represented a mix of taxa that dominated the microbiome but were less diverse than CST3.

In addition, when analyses were carried out separately for CST 4, 5, and 6, we saw no differences in their modifying influences on the associations between antecedent or postonset yeast infections, a history of anxiety, or a history of mood disorder and vulvodynia. CST1 and CST2 were kept separate because both groups are dominated by a single *Lactobacillus* species. CST1 was most closely related to *Lactobacillus crispatus*. CST2 was identified as *L iners*. The association with antecedent yeast infections was largely confined to women with CST1 and CST2 (Figure 3). There did not appear to be a specific CST associated with anxiety, but CST2 was more aligned with seeing an association between antecedent depression and vulvodynia.

Lastly, analysis using ALDEx2[22] (Supplemental Appendix) showed no statistically significant associations of

FIGURE 3
Age-adjusted odds ratios and 95% confidence intervals for antecedent yeast infections



Age-adjusted odds ratios and 95% confidence intervals for antecedent yeast infections **(A)**, Please confirmpostonset yeast infections **(B)**, a history of anxiety **(C)**, and a history of mood disorder **(D)**. For the yeast infections, zero was used as the reference. Vulvodynia cases (n = 215) and matched controls (n = 222), 2010–2015.

CST, community state type.

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individual taxa abundance by vulvodynia status or community state type.

Comment

Principal findings

There was no overall microbiome differences between cases and controls. However, we observed associations between vulvodynia and history of the following: (1) antecedent yeast infections and (2) psychosocial risk factors such as a history of childhood abuse, antecedent anxiety, mood disorders, and higher levels of rumination only among women with vaginal microbiomes that have alpha diversity below the median (low) but not alpha diversity above the median (high) as measured using the Shannon Diversity Index, an index that takes into account the number of species and their relative abundance. Because the microbiome was measured at the time of vulvodynia and a comparable time period in controls, it is uncertain whether vaginal microbiome diversity modifies the association between these risk factors and vulvodynia, or becomes altered as a consequence of these associations.

Disease states such as bacterial vaginosis are associated with higher diversity of the vaginal microbiome.[23] However, vaginal diversity was not associated with vulvodynia, but instead modified the associations between vulvodynia and previously studied risk factors. Most studies to date have focused on the impact of infectious diseases on the microbiome and are not comparable with the study of a multifactorial disease such as vulvodynia.[24–26] The study by Jayaram et al[11] identified *L iners* to be more prevalent and abundant in women with vulvar vestibulitis syndrome, whereas in controls *L crispatus* was more prevalent and abundant. The study by Vadala et al[12] reported less lactobacillus species in cases compared with controls. However, neither

study directly evaluated alpha diversity or community state types.

Clinical implications

It is too soon to speculate on the clinical implications of these findings. Women with vulvodynia are likely having less frequent sexual activity, which has been suggested to be associated with a less diverse vaginal microbiome.[27] However, we did not observe an association between microbiome diversity and vulvodynia. Instead, we saw that chronic yeast infections and psychosocial risk factors were strongly associated with vulvodynia only among women with less diverse vaginal microbiomes. Our data suggest that a more diverse microbiome environment may mitigate the deleterious effects of chronic infections and psychosocial morbidity shown to be associated with vulvodynia.

Research implications

Alpha diversity captures the number of species present and their relative abundance and is related to, but does not give specific information on, the emergent properties of a microbial community, such as stability.[28] Thus, the modifying effect of diversity on psychosocial risk factors should be seen as a starting point rather than an explanation per se.[28]

Both high- and low-diversity populations have been associated with stability and healthy and diseased states. For example, over the course of a healthy pregnancy, the vaginal microbiota become less diverse.[29] This is thought to optimize colonization of the infant during labor and delivery, but because these less diverse communities tend to be predominated by acid-producing bacteria (eg, *Lactobacillus* species), it might also limit growth of pathogens.[29] By contrast, bacterial vaginosis is characterized by a highly diverse vaginal microbial community and a higher pH.

A limitation of our measure of diversity, based on sequencing data, is that it does not measure the absolute abundance of taxa present. That is, the same diversity can be present with dense biomass, as might be found in women with and without bacterial vaginosis. However, the quantity of selected organisms identified using culture techniques (expressed as $\log_{10}$ colony-forming units per gram of vaginal secretions) did not differ among those with low- and high-diversity microbiomes.

Because there were no single taxa or groups of taxa associated with vulvodynia, our findings should stimulate us to look for and identify factors that might decrease vaginal diversity. Several hypotheses come to mind. First, women with repeated yeast infections also are more likely to have atopic diseases (eczema, allergic rhinitis, and asthma), suggesting heightened local innate immunity, which may result in changes in the vaginal microbiota.[30] *Candida* species are also human commensals; the majority of women are colonized with *Candida* species at 1 or more times in their lives, usually without symptoms.[31] Thus, *Candida* species may play an important role in the development and maintenance of normal mixed species biofilms found in the vaginal cavity.[31]

Second, women with multiple yeast infections were most likely treated with multiple courses of antifungals, and they often follow urinary tract infections, which are treated with antibiotics. Antifungals and antibiotic treatment can disrupt the vaginal microbiota.[31]

Third, the change in diversity may be mediated by infection with a bacteriophage (viruses that infect bacteria) known to be important mediators of microbial communities in other systems.[32]

Strengths and limitations

Our study used clinically confirmed cases of vulvodynia and general population controls. Additionally, we assessed temporality with respect to the explored risk factors using interview questions targeted to before and after onset of vulvodynia (cases) or a comparable reference age (controls). A further strength is the collection of orthogonal data including both standard culture-based microbiological methods and newer sequencing-based methods for quantitation of bacterial taxa.

This study is limited in its interpretability because of the lack of, and timing of, the vaginal microbiome sample. The vaginal community was assessed at the time of vulvodynia and probably reflects a combination of response to the disease process and the ongoing processes that maintain symptoms. Because vaginal samples were taken after the onset of vulvodynia, we cannot make conclusions on the temporality of associations seen, particularly with respect to the onset of vulvodynia. Participants were asked to recall the onset of certain conditions as either before or after vulvar pain onset or reference age, which could result in recall bias. However, Harlow et al[8] used quantitative bias analyses to evaluate the possibility of recall bias in this population and suggested that the observed associations for antecedent yeast infections were likely an underestimate of the true effect, except under less plausible scenarios of perfect specificity and no false-positive yeast infections among women with no history of vulvar pain.

Additionally, this study was conducted in premenopausal women 18−40 years of age who were largely restricted to those self-reported as white. Thus, we could not evaluate the contribution of these findings to postmenopausal women or to ethnic diversity, which has been shown to be a factor influencing the vaginal microbiome.[33]

Lastly, the size of our data set did not allow us to assess more than a dichotomous level of effect measure modification by microbiome diversity. Nevertheless, we pose a hypothesis that we hope others will assess within larger and more temporally appropriate data sets.

Conclusions

We evaluated the vaginal microbiome in a large, well-characterized population of women with and without vulvodynia and found no overall differences. However, we found strong and significant associations between vulvodynia and known risk factors (eg, yeast infections, childhood abuse, anxiety, and mood disorders) in women with low-diversity vaginal microbiomes, and these associations were not attenuated when controlling for other risk factors. ■

Acknowledgments

Contribution to authorship include the following: Ms Bedford carried out all analyses and drafted

the manuscript under the advice and guidance of Dr Parker, Dr Foxman, and Dr Harlow. Dr Parker assisted in the analysis and interpretation of the findings. Ms Davis and Salzman carried out bioinformatics and laboratory analyses. Dr Hillier completed all the culture-based assessments and assisted in the editing and interpretation of the findings. Drs Foxman and Harlow secured the funding for the study, jointly supervised all aspects of the molecular assessments, analyses, interpretation, and presentation for the manuscript. This study was approved by the Human Subjects Research Committee at the University of Minnesota. Written informed consent was obtained from all participating women. The University of Minnesota's Institutional Review Board most recent approval of continuing review was Jan. 2, 2019. Main study approval was Sept. 29, 2009.

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Supplemental Appendix

Culture based methods

a. Vulvovaginal swab samples and procedures for microbiological assessments

Specimens were collected using a duplicate swab sampling technique in which 2 swabs are inserted simultaneously into the posterior vagina, taking care to avoid contact with external genitalia and other sources of contamination. The swabs were rotated several times along the upper lateral third of the vaginal vault to saturate the cotton tip, and both swabs were removed. A total of 4 swabs were collected using this technique. The first swab was preweighed and used for an estimation of sample weight, and the second swab was processed for recovery of microorganisms. These 2 swabs were collected simultaneously with the preweighed swab and stored in a sterile glass vial, and the second swab was placed into Amies transport medium without charcoal. The third and fourth swabs were collected simultaneously, and swab 3 was used for measuring pH, performing the Whiff test, and making a smear for Nugent score determination. The fourth swab was collected and stored at −80°C for future assessments.

The swabs and slide were transferred and processed within 24 hours. The preweighed swab and tube were reweighed and the difference recorded as the sample weight. The swab sample was passed into an anaerobic chamber and agitated on a vortex mixer for 3−5 minutes until the sample was completely dispersed. Serial dilutions of the sample were made in phosphate-buffered saline and the undiluted sample, as well as aliquots of each dilution, was plated onto various selective and nonselective media. The culture media for recovering anaerobes was prereduced brucella-base agar with 5% sheep blood enriched with hemin and vitamin K_1 (BMB) and prereduced brucella-base agar with 5% laked sheep blood, 100 μg of kanamycin, and 7.5 μg of vancomycin per milliliter and supplemented with hemin and vitamin K_1 (BKV). Media for recovery of facultative anaerobes was 5% sheep blood in tryptic soy agar (TSA), MacConkey agar (MAC), and Sabouraud dextrose (SABDEX). Chocolate agar (CHOC) was used for the recovery of *Gardnerella vaginalis*. A-7 is used for the recovery of *Mycoplasma* and *Ureaplasma*. BMB, BKV, and A-7 plates are incubated in an anaerobic chamber for a minimum of 120 hours at 35°C before enumeration. TSA, MAC, and SABDEX plates are incubated in air and CHOC plates in 5% carbon dioxide for 48 hours. Following incubation the various colony types were enumerated, isolated, and identified using established criteria. All estimates of the bacterial population size are expressed as $\log_{10}$ colony-forming units per gram of vaginal secretions ($\log_{10}$ CFU/g).

b. Identification of microorganisms

Following incubation under appropriate atmospheric conditions, colonies were counted on the various media and individual colony types selected for identification, based on colony morphology. *Enterobacteriaceae* were identified using the AP 20E system or by long-chain fatty acid analysis using the MIS system (MIDI; Microbial Identification Inc, Newark, DE). Catalase positive, Gram-positive, pleomorphic rods are classified as *Corynebacterium* sp. Aerobic, Gram-positive spore-forming rods are identified as *Bacillus* sp catalase-positive, coagulase-positive, Gram-positive cocci are identified as staphylococcus aureus, while coagulase-negative cocci are classified as staphylococcus species. Catalase-negative, Gram-positive cocci are categorized as Streptococcus and further identified using the api20 strep (bioMerieux-USA, Hazelwood, MO). Gram-positive, or Gram-variable catalase negative rods showing beta hemolysis on HBT medium are identified as *G vaginalis* using a rapid identification kit (Austin Biologicals Labs, Austin, TX). Gas chromatographic analysis of glucose fermentation products was used for preliminary identification of obligate anaerobes and Gram-positive, catalase-negative, facultative rods (lactobacilli). Further identification to species level was done with the Anastat II system (Innovative Diagnostics Systems, Norcross, GA) or the MIS long-chain fatty acid system. All counts are recorded as $\log_{10}$ CFU per gram of sample (127,128).

We evaluated the association between white blood cells (WBC) count, Nugent score, Gram stain score, and quantitative culture results. WBCs were scored as an average of 5 nonadjacent fields of view, with 0 indicating no WBC found in any field and 1 indicating there was a single WBC in 1 or all of the 5 fields of view. The Nugent score was calculated by counting the relative proportion of bacterial morphotypes (large Gram-positive rods, small Gram-negative or variable rods, or curved rods), with a score of 0 corresponding to the most *Lactobacillus*-predominant vaginal flora and a score of 10 corresponding to a vaginal flora characterized by replacement of lactobacilli by *Gardnerella*, anaerobic Gram-negative rods (*Prevotella*, *Porphyromonas*, *Bacteroides*), and *Mobiluncus* (curved rods) morphotypes. A Nugent score from 0 to 3 is considered normal, 4 to 6 intermediate and 7 to 10 bacterial vaginosis. Quantitative vaginal cultures were recorded as $\log(3 \times 10^Z)$ colony-forming units per milliliter.

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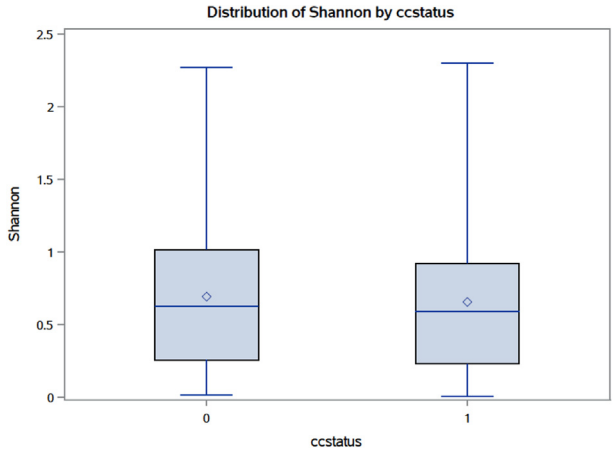
SUPPLEMENTAL FIGURE 1
Shannon (alpha) diversity



Vulvodynia cases (n = 215) and matched controls (n = 222), 2010–2015

CST, community state type.

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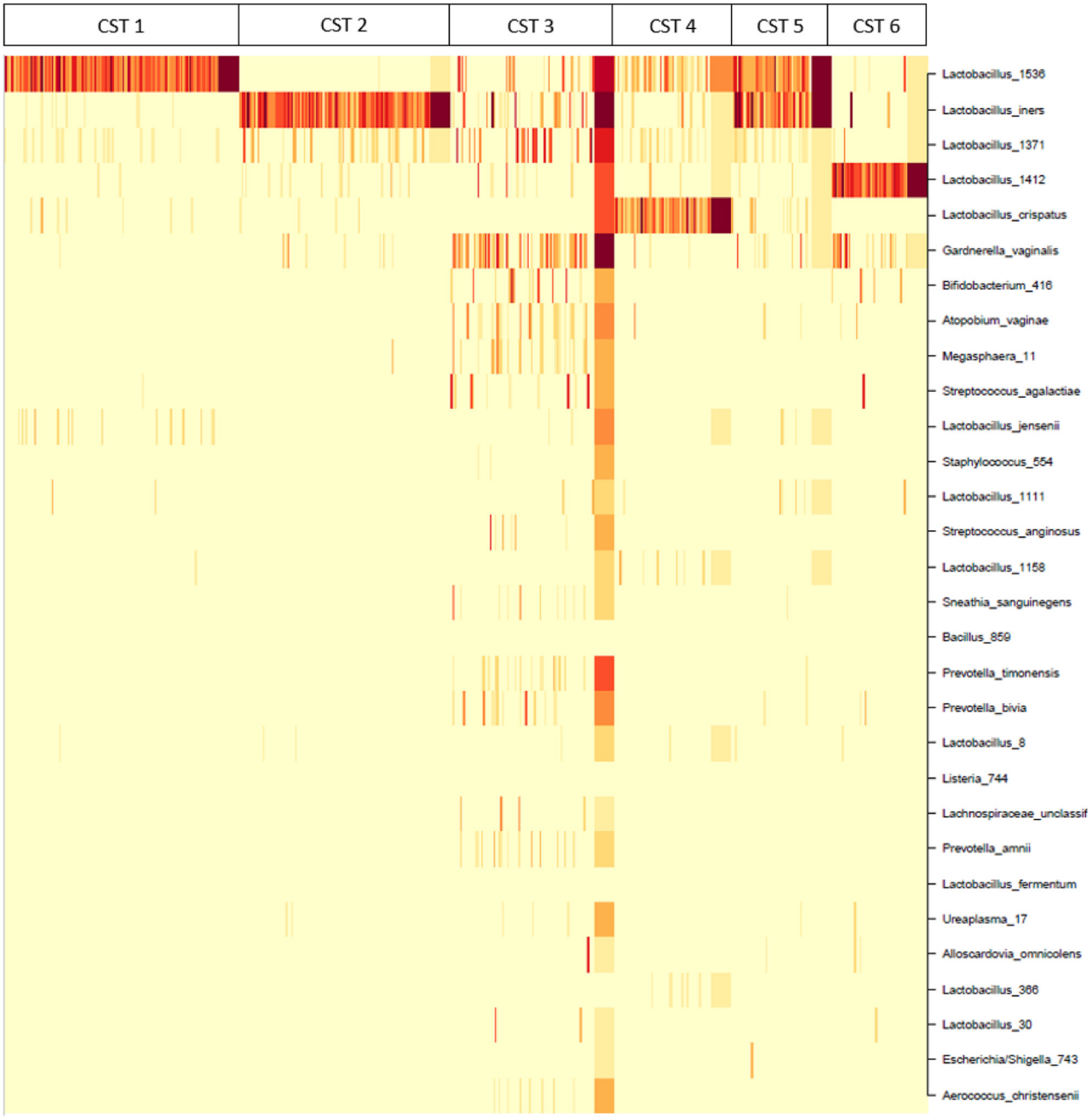
SUPPLEMENTAL FIGURE 2
Heatmap of read counts showing top 30 taxa



Narrow columns are samples and the color represents square root counts. We can determine the dominant species for each community type by looking at the thicker bands with darker colors. CST1: *Lactobacillus* 1536; CST 2: *L iners*; CST 3: diverse, and *Gardnerella vaginalis*; CST4: *L crispatus* and *Lactobacillus* 1536 codominant; CST 5: *Lactobacillus* 1536 and *L iners* codominant; CST6: *Lactobacillus* 1412. Vulvodynia cases (n = 215) and matched controls (n = 222), 2010–2015.

CST, community state type.

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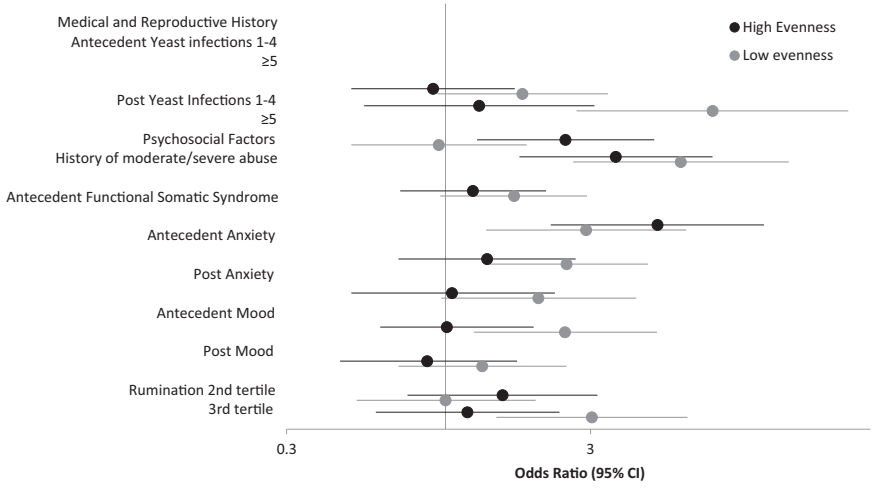
SUPPLEMENTAL FIGURE 3



Age-adjusted odds ratios for yeast infections and psychosocial factors stratified by evenness. Antecedent and postonset categories were defined as before or after the onset of vulvodynia in cases and before or after reference age in controls. Vulvodynia cases (n = 215) and matched controls (n = 222), 2010–2015.

CI, confidence interval.

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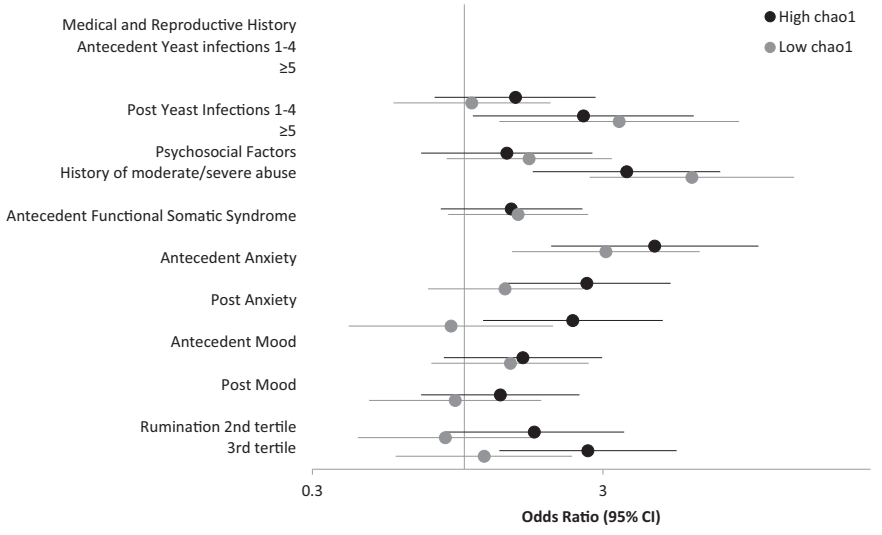
SUPPLEMENTAL FIGURE 4



Age-adjusted odds ratios for yeast infections and psychosocial factors stratified by richness (assessed using Chao1). Antecedent and postonset categories were defined as before or after the onset of vulvodynia in cases and before or after reference age in controls. Vulvodynia cases (n = 215) and matched controls (n = 222), 2010–2015.

CI, confidence interval.

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SUPPLEMENTAL TABLE 1

Culture-based and slide-based microbiological features, vulvodynia cases (n = 215) and matched controls (n = 222), with age-adjusted odds ratios and 95% confidence intervals, 2010–2015

Variables	Cases n (%) 215 (49.2)	Controls n (%) 222 (50.8)	OR (95% CI)
WBC count (WBCs/field)			
0	40 (18.6)	38 (17.1)	1.00
≤1	68 (31.6)	58 (26.1)	1.0 (0.58–1.9)
2–4	68 (31.6)	85 (38.3)	0.65 (0.37–1.2)
5–30	29 (13.5)	36 (16.2)	0.65 (0.33–1.3)
Nugent score			
Normal	167 (77.7)	176 (79.3)	1.00
Intermediate	26 (12.1)	26 (11.7)	1.3 (0.69–2.3)
Bacterial vaginosis	12 (5.6)	15 (6.8)	0.99 (0.44–2.2)
Quantitative vaginal culture results[a]			
Lactobacillus, H_2O_2 negative			
0	144 (67.0)	151 (68.0)	1.00
Low	31 (14.4)	33 (14.9)	1.1 (0.61–1.9)
High	32 (14.9)	34 (15.3)	0.97 (0.56–1.7)
Lactobacillus, H_2O_2 positive			
0	27 (12.6)	41 (18.5)	1.00
Low	91 (42.3)	87 (39.2)	1.6 (0.87–2.8)
High	89 (41.4)	90 (40.5)	1.3 (0.74–2.4)
Gardnerella vaginalis			
0	146 (67.9)	152 (68.5)	1.00
Low	26 (12.1)	32 (14.4)	0.71 (0.39–1.3)
High	28 (13.0)	30 (13.5)	1.1 (0.62–2.0)
Enterococcus			
0	163 (75.8)	178 (80.2)	1.00
Low	17 (7.9)	18 (8.1)	1.3 (0.61–2.6)
High	20 (9.3)	18 (8.1)	1.3 (0.65–2.6)
Escherichia coli			
0	188 (87.4)	195 (87.8)	1.00
Low	5 (2.3)	10 (4.5)	0.50 (1.66–1.5)
High	7 (3.3)	9 (4.1)	0.84 (0.30–2.4)
Viridans *Streptococcus* species			
0	127 (59.1)	153 (68.9)	1.00
Low	14 (6.5)	21 (9.5)	0.79 (0.38–1.6)
High	12 (5.6)	24 (10.8)	0.66 (0.31–1.4)

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SUPPLEMENTAL TABLE 1

Culture-based and slide-based microbiological features, vulvodynia cases (n = 215) and matched controls (n = 222), with age-adjusted odds ratios and 95% confidence intervals, 2010–2015 *(continued)*

	Cases	Controls	
	n (%)	n (%)	
Variables	215 (49.2)	222 (50.8)	OR (95% CI)
Group B beta *Streptococcus*			
0	179 (83.3)	196 (88.3)	1.00
Low	10 (4.7)	8 (3.6)	1.2 (0.44–3.1)
High	11 (5.2)	10 (4.5)	1.3 (0.52–3.1)
Aerobic GNR			
0	196 (91.2)	209 (94.1)	1.00
Low	1 (0.47)	1 (0.45)	1.9 (0.11–30.4)
High	3 (1.4)	4 (1.8)	0.80 (0.17–3.7)
Anaerobic GNR, black pigmented			
0	176 (81.9)	188 (84.7)	1.00
Low	2 (0.9)	2 (0.9)	0.85 (0.12–6.3)
High	22 (10.2)	24 (10.8)	1.1 (0.60–2.1)
Anaerobic GNR, nonpigmented			
0	141 (65.6)	149 (67.1)	1.00
Low	23 (10.7)	27 (12.2)	0.88 (0.47–1.6)
High	36 (16.7)	38 (17.1)	1.2 (0.68–2.0)

CI, confidence interval; *GNR*, Gram-negative bacilli; *OR* odds ratio; *WBC*, white blood cells.

[a] Low/high was determined by median count of all nonzeros (counts are log[3 × 10ˆZ CFU per milliliter]).

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SUPPLEMENTAL TABLE 2

OR (95% CI) for the association between vulvodynia and antecedent yeast infections stratified by alpha diversity and adjusted using multiple models, vulvodynia cases (n = 215) and matched controls (n = 222), 2010–2015

Variables	High alpha diversity (n =106 cases, 111 controls)		Low alpha diversity (n =109 cases, 111 controls)	
Antecedent yeast infections	1-4	>5	1-4	>5
Cases, n (%)	30 (28.3)	13 (12.3)	32 (29.4)	22 (20.2)
Age adjusted	0.82 (0.45–1.5)	1.2 (0.50–2.9)	2.0 (1.1–4.0)	8.1 (2.9–22.7)
Model 2[a]	0.81 (0.44–1.5)	1.2 (0.48–2.8)	2.0 (1.0–3.9)	7.2 (2.5–20.6)
Model 3[b]	0.81 (0.43–1.5)	1.2 (0.48–2.9)	1.8 (0.9–3.5)	6.9 (2.4–19.8)
Model 4[c]	0.84 (0.46–1.5)	1.2 (0.50–2.9)	2.0 (1.0–3.8)	7.9 (2.8–22.7)
Fully adjusted[d]	0.83 (0.44–1.6)	1.2 (0.47–2.9)	1.8 (0.9–3.6)	6.9 (2.3–20.1)

CI, confidence interval; *OR*, odds ratio.

[a] Model 1 plus antecedent anxiety; [b] Model 1 plus antecedent mood; [c] Model 1 plus childhood abuse; [d] Model 1 plus antecedent anxiety, antecedent mood, and childhood abuse.

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SUPPLEMENTAL TABLE 3

OR (95% CI) for the association between vulvodynia and antecedent anxiety and mood disorders stratified by alpha diversity and adjusted using multiple models, vulvodynia cases (n = 215) and matched controls (n = 222), 2010–2015

Variables	Antecedent anxiety		Antecedent mood	
	High alpha (n = 106 cases, 111 controls)	Low alpha (n = 109 cases, 111 controls)	High alpha (n = 106 cases, 111 controls)	Low alpha (n = 109 cases, 111 controls)
n (%) of cases	25 (23.6)	43 (39.5)	34 (32.1)	32 (29.4)
Age adjusted	1.2 (0.64–2.4)	2.8 (1.5–5.3)	1.0 (0.57–1.8)	2.8 (1.4–5.6)
Model 2[a]	1.2 (0.61–2.3)	2.5 (1.3–4.7)	1.0 (0.56–1.9)	2.1 (1.0–4.4)
Model 3[b]	1.1 (0.57–2.2)	2.8 (1.5–5.2)	1.0 (0.52–1.7)	2.5 (1.2–5.1)
Fully adjusted[c]	1.1 (0.56–2.1)	2.4 (1.2–4.7)	1.0 (0.53–1.9)	1.9 (0.89–4.1)

UTI, urinary tract infection.

[a] Adjusted for age and antecedent yeast infections; [b] Adjusted for age and a history of UTI; [c] Adjusted for age, antecedent yeast infections, and a history of UTI.

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