Appendix S1

Culture based methods:

a. *Vulvovaginal swab samples and procedures for microbiological assessments*

Specimens were collected using a duplicate swab sampling technique where two 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 four swabs were collected using this technique. The first swab was pre-weighed and used for an estimation of sample weight and the second swab was processed for recovery of microorganisms. These two swabs were collected simultaneously with the pre-weighed swab and stored in a sterile glass vial and the second swab was placed into Amies transport medium without charcoal. The 3rd and 4th 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 4th swab was collected and stored at -80^{o}C for future assessments.

The swabs and slide were transferred and processed within 24 hours. The pre-weighed 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 to 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 pre-reduced 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 ml, 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 h at 35^{o}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 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/gm of sample (127,128).

We evaluated the association between WBC count, Nugent Score, Gram Stain Score, and quantitative culture results. White blood cells (WBCs) were scored as an average of 5 non-adjacent 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 0-3 is considered normal, 4-6 intermediate and 7-10 Bacterial Vaginosis. Quantitative vaginal cultures were recorded as log(3 x 10^Z) colony forming units/ml.

Table S1. 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.

		Cases	**Controls**	**OR (95% CI)**
		N (%)	N (%)	
		215 (49.2)	222 (50.8)	
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[1]				
***Lactobacillus*, H2O2 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*, H2O2 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)
E. 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)
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[2]	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, non-pigmented	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)

[1]Low/High determined by median count of all non-zeros (counts are log(3x10^Z CFU/ml))

[2]GNR: gram-negative bacilli

Table S2. 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.

	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)

[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

Table S3. 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.

	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)

[a]Adjusted for age and antecedent yeast infections

[b]Adjusted for age and history of UTI

[c]Adjusted for age, antecedent yeast infections, history of UTI

Figure S1: Shannon (alpha) diversity. Vulvodynia cases (n=215) and matched controls (n=222), 2010-2015.

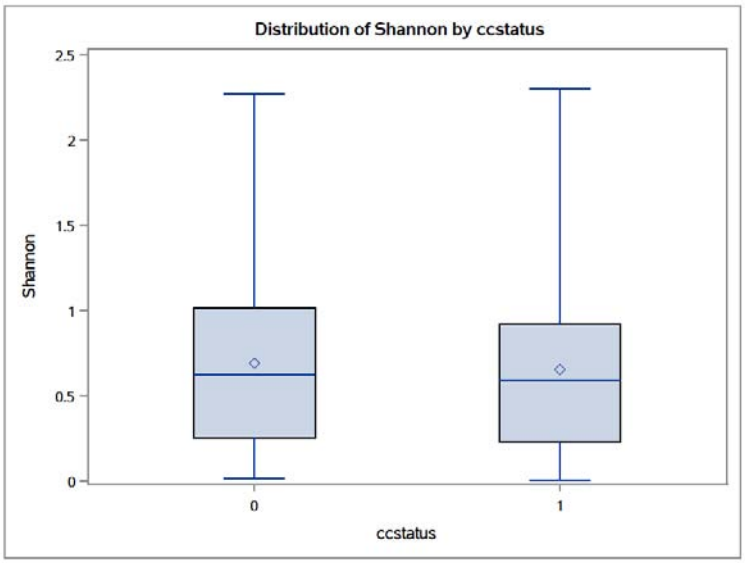

Figure S2: Heatmap of read counts showing top 30 taxa. Narrow columns are samples and the color represents square-root counts. We can determine the dominant species for each community type by looking at the thicker bands with darker colors. CST1: Lactobacillus_1536; CST 2: *L. iners*; CST 3: Diverse, and Gardnerella_vaginalis; CST4: *L. crispatus* and Lactobacillus_1536 co-dominant; CST 5: Lactobacillus_1536 and *L .iners* co-dominant; CST6: Lactobacillus_1412. CST=community state type. Vulvodynia cases (n=215) and matched controls (n=222), 2010-2015.

Figure S3. Age-adjusted odds ratios for yeast infections and psychosocial factors stratified by evenness. Antecedent and post-onset categories 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.

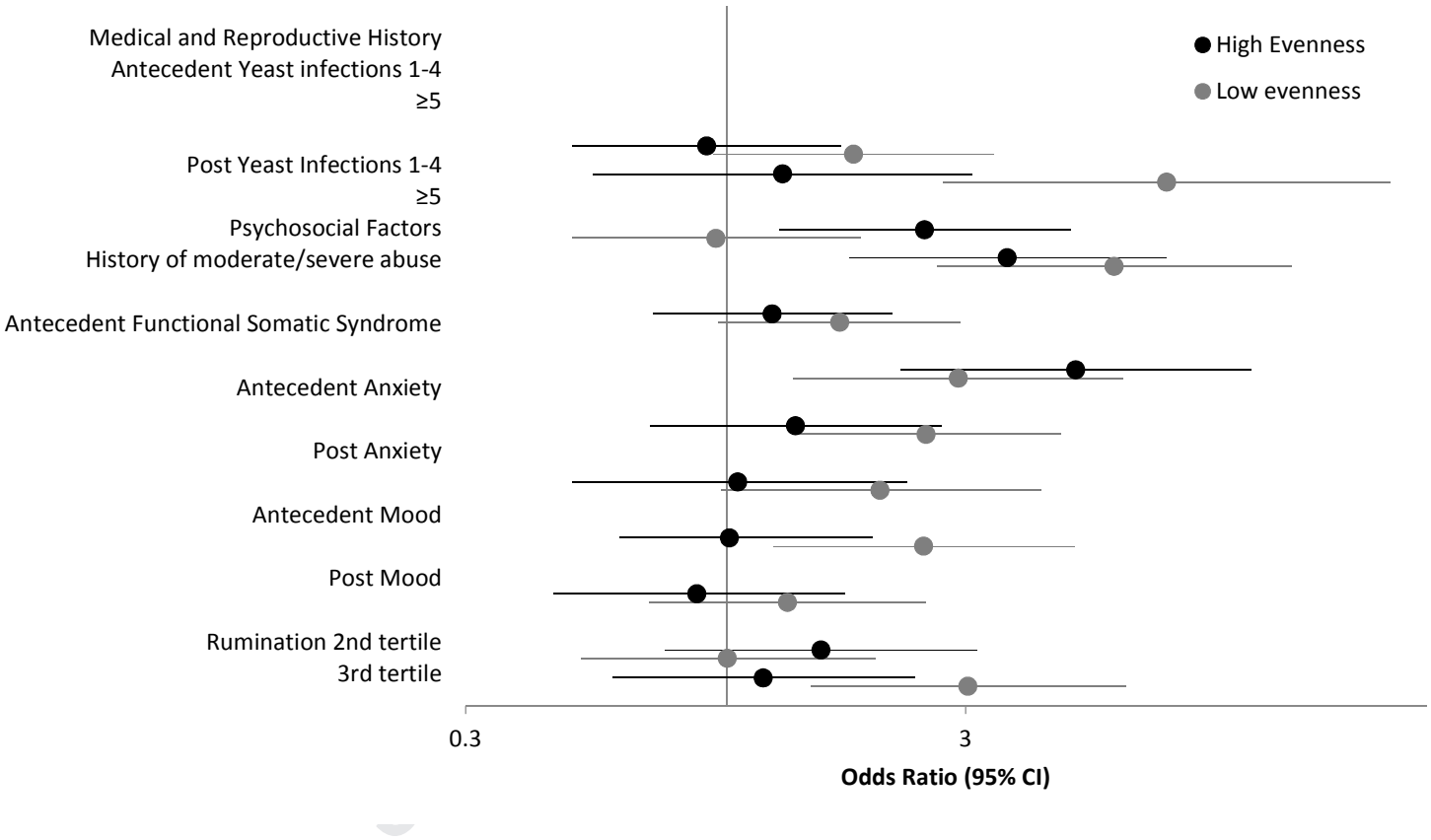

Figure S4. Age-adjusted odds ratios for yeast infections and psychosocial factors stratified by richness (assessed using Chao1). Antecedent and post-onset categories defined as before or after the onset of vulvodynia in cases and before or after reference age in controls. Vulvodynia cases (n=215) and matched controls (n=222), 2010-2015.