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Vaginal microbiota and genitourinary menopausal symptoms: A cross sectional analysis

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Abstract

Objective—To examine associations between the composition of the vaginal microbiota and genitourinary menopausal symptoms, serum estrogen, and vaginal glycogen.

Methods—For this cross-sectional study, 88 women ages 40–62 enrolled in a hot flash treatment trial provided vaginal swabs and a blood sample at enrollment. Bacterial communities were characterized using 16S rRNA PCR and deep sequencing targeting the V3-V4 region. Quantities of *Lactobacillus crispatus* and *L. iners* were measured using qPCR. Self-reported genitourinary symptoms included: 1) presence and severity of individual symptoms and 2) identification of most bothersome symptom. Glycogen was measured fluorometrically in swab eluate. Serum estradiol

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(E2) and estrone (E1) were measured by liquid chromatography/mass spectrometry. Associations between bacteria, symptoms, glycogen, and serum estrogens were tested by linear regression or Wilcoxon signed-rank test, adjusted for multiple comparisons. Comparisons between groups used Kruskall-Wallis or Fisher's exact test.

Results—Of the 88 women, 33 (38%) had a majority of *Lactobacillus* species, while 58 (66%) had any *Lactobacillus* detected. Over half (53%) reported ≥ 1 vulvovaginal symptom (most commonly dryness), but symptoms were not associated with the presence of *Lactobacillus* species. Women with *Lactobacillus* dominant communities had higher unconjugated serum estrone, but no difference in vaginal glycogen levels, compared to those with non-*Lactobacillus* dominant communities. Higher serum E2 and E1 were not associated with higher vaginal glycogen, nor detection of individual genera.

Conclusions—Presence of *Lactobacillus*-dominant vaginal microbiota was not associated with fewer vulvovaginal symptoms. Serum estrone was higher in women with *Lactobacillus*-dominance, but vaginal free glycogen was not associated with composition of the vaginal microbiota.

Keywords

Menopause; vaginal microbiota; vaginal glycogen; genitourinary symptoms of menopause

Introduction

Genitourinary symptoms of menopause (GSM) including vaginal dryness and discomfort occur in 45–77% of women and cause significant distress.[1,2] Few studies have evaluated risk factors for vaginal symptoms in menopausal women. In a study of 32 postmenopausal women, those with greater evidence of genitourinary atrophy on exam had lower abundance of *Lactobacillus species*, and a more diverse community of vaginal microbes.[3] However, overall severity of patient-reported symptoms was significantly lower than severity of observed atrophy, and did not correlate well with exam findings.

Studies using both culture and molecular methods have shown that postmenopausal women are less likely than premenopausal women to have vaginal colonization with *Lactobacillus* bacterial species.[3–5] This has been attributed to decreased serum estrogen, which reduces glycogen content in vaginal epithelial cells, and limits the energy source for lactobacilli. While all postmenopausal women experience a drop in serum estradiol, not all lose vaginal lactobacilli.[3,4,6] Recent data have shown that free glycogen in vaginal fluid, which is liberated from epithelial cells by enzymes like α-amylase, is associated with *Lactobacillus* colonization in both pre- and postmenopausal women, suggesting glycogen may be a mediating factor for *Lactobacillus* presence.[7,8] In premenopausal women, serum estrogen levels were not correlated with free vaginal glycogen levels, but this relationship has not been examined in postmenopausal women.[9]

To evaluate the role of these factors in genitourinary symptoms of menopause we conducted a pilot analysis of the association between vaginal microbiota, systemic hormones, vaginal glycogen and vulvovaginal symptoms in postmenopausal women.

Methods

Study participants

This pilot study used pre-treatment samples and data from a subset of women who participated in a 3-arm double-blind randomized trial of oral estradiol, venlafaxine and placebo for the alleviation of menopausal hot flashes. The study was conducted at three Menopause Strategies: Finding Lasting Answers for Symptoms and Health (MsFLASH) network sites (Boston, Philadelphia and Seattle). Details about the MsFLASH Research Network,[10] study design, methods, and main trial results have been reported elsewhere.[11] Women aged 40–62, in the menopausal transition (≤12 months since last menstrual period) or in menopause (> 12 months since last period), who reported ≥ 14 hot flashes per week and met other inclusion criteria participated. The protocol was approved by the appropriate institutional review board at each site. All participants provided written informed consent. Women who agreed to the ancillary vaginal microbiome study signed a second consent form. The parent study enrolled 339 women between November 2011 and October 2012. Enrollment in the vaginal health substudy was offered between June and October 2012 and 93 of 117 (79%) women who enrolled in the parent study during that time also enrolled in the vaginal health substudy.

Demographic and symptom measures

Baseline demographic characteristics were assessed by questionnaire and included: smoking status, menopausal status (menopause transition, postmenopause), and health status. Validated questionnaires at baseline also evaluated menopause quality of life (MENQOL),[12] depressive symptoms (9-item scale from the Patient Health Questionnaire [PHQ-9]),[13] anxiety (7-item Generalized Anxiety Disorder scale [GAD-7]),[14] and sexual function (19-item Female Sexual Function Index [FSFI]).[15] Height and body weight were measured at baseline and were used to calculate body mass index (BMI). There are no validated scales for measurement of genitourinary symptoms of menopause, therefore we used two instruments based on previous studies.[16,17] Self-reported vaginal symptom measures included: 1) presence and severity of individual symptoms (vaginal dryness, vulvovaginal itch/burn, vaginal discharge, vaginal pain with intercourse) on a 5-point scale (ranging from mild to severe) and 2) selection of the most bothersome symptom for the participant. Women had blood drawn and self-collected vaginal swabs at enrollment.

Characterization of the vaginal microbiota

Vaginal swabs were mailed to the Fredricks Lab from study sites via regular mail and stored at −80C until processed, as previously described.[18] Swabs were eluted in 400 μL of sterile, filtered saline and centrifuged at 14,000 rpm for 10 minutes. DNA was extracted from the cell pellet using the MoBio Bacteremia extraction kit (MoBio, Carlsbad, CA) as previously described.[18] The bacterial 16S rRNA gene was amplified using primers for the V3-V4 hypervariable region, libraries were created using barcoded primers, and amplicons were sequenced using the Roche 454 Titanium platform (Roche, CT).[19] Sequence reads have been deposited to the NCBI Short Read Archive (SRP100779). Negative controls included sham digests that were processed in the same way as samples to assess contamination from DNA extraction or PCR reagents. Sequences were filtered for length (minimum 250 bp) and

quality score (minimum 30), and reads originating from contaminants in PCR controls were removed. Sequence reads were classified using the *pplacer* phylogenetic placement tool and a curated reference set of vaginal bacterial sequences.[19] Based on the dominant bacterial genus, participants were categorized into two groups: *Lactobacillus*-dominant (> 50% of sequences from *Lactobacillus* species) and non-*Lactobacillus* dominant. For quantification of *L. crispatus* and *L. iners*, species-specific qPCR using a TaqMan-based assay was performed as previously described.[18,20]

Vaginal glycogen assay

Vaginal swabs were vortexed in 350 μL filtered saline. The recovered vaginal fluid was further diluted in saline (1:5), and 50 μL of the diluted fluid was used in a fluorometric assay (BioVision, Milpitas, CA) to measure glycogen levels.

Serum estrogen measurements

At each clinic visit blood was drawn and serum stored at −80C until processing. An ultrasensitive stable isotope dilution liquid chromatography/selected reaction monitoring/mass spectrometry (LC/SRM/MS) assay was used to measure total and unconjugated estradiol and estrone.[21] The limit of detection for each estrogen using 0.5 mL of serum was 0.156 pg/mL and linear standard curves were obtained up to 20 pg/mL.

Statistical analysis

For the cross-sectional analysis, associations between individual bacterial taxa, estrogen and glycogen were estimated by linear regression, and with symptoms by Wilcoxon signed-rank test, both adjusted for multiple comparisons via Bonferroni correction. Comparisons of participant characteristics between women with and without *Lactobacillus*-dominant microbiota used Student's t-test, Wilcoxon signed-rank test, chi square or Fisher's exact test, as appropriate. Similar comparisons were performed to assess whether characteristics of the substudy cohort were similar to the parent cohort. Symptoms were categorized for analysis into None (not present), Mild (score 1–2 on 5–point scale) or Moderate-severe (score 3–5).

Results

We enrolled 93 women in the vaginal health substudy of whom 88 (95%) had complete results for evaluation and were included in the cross-sectional analysis. The substudy cohort was 55% white and 39% African American, had mean (± standard deviation) age of 54 ± 4 years, BMI of 29 ± 6 kg/m^2 and MENQOL score of 3.6 ± 1.2. None of these characteristics significantly differed from the remainder of the parent cohort (data not shown). Most women (71/88, 81%) were postmenopausal while 17/88 (19%) had a period within the last 12 months and were in the late menopausal transition. Over half (47/88, 53%) reported at least one of the evaluated symptoms of any severity (vaginal dryness (38%), vulvovaginal itch/burn (25%), vaginal discharge (26%), vaginal pain with insertion (10%)). Of the 44 women reporting sex with a male or female partner in the past month, 7 (16%) reported vaginal pain with insertion, in contrast to 1/19 (5%) reporting only self-stimulation and 1/22 (5%) reporting no sexual activity at all.

By 16S rRNA gene sequencing of amplicons, 58 (66%) women had any *Lactobacillus* species detected and 33 (38%) had a *Lactobacillus*-dominant vaginal microbiota (Figure 1). *Lactobacillus* dominance was not associated with severity of individual symptoms (Figure 2). By species-specific qPCR, 21 (24%) had both *L. crispatus* and *L. iners*, 8 (9%) had *L. crispatus* only, 28 (32%) had *L. iners* only and 30 (34%) had neither species detected. By qPCR there was no significant difference in detection or quantity of *L. crispatus* and *L. iners* between women reporting no, mild or moderate-severe symptoms (data not shown). When analyzed by individual bacterial taxa, women with vaginal itching (n = 10) or pain (n = 7) were more likely to have BV Associated Bacterium 1(BVAB1) ($p<0.01$). The distribution and severity of the most bothersome vaginal symptom did not differ significantly between women with and without a *Lactobacillus*-dominant vaginal microbiota (Table 1). In contrast to most studies of pre-menopausal women, we saw no difference in ethnicity between women with *Lactobacillus*-dominance and those without.

Median unconjugated serum estrone was higher in women with a *Lactobacillus* dominant compared to women with a non-*Lactobacillus* dominant microbiota (Table 1, $p = 0.007$). There were no significant associations between levels of unconjugated estrone and quantity of *L. crispatus* or *L. iners (*Figure 3). There were no significant differences in serum estradiol between women with majority *Lactobacillus* sequences vs. those with other species. There was no association between serum estrogens and report of symptoms (data not shown). In addition, there were no significant associations between serum estradiol or estrone levels and individual bacterial taxa after controlling for multiple comparisons.

There was no significant association between serum estrogens and vaginal glycogen levels. Vaginal glycogen levels did not significantly differ between women with a *Lactobacillus* dominant microbiota and those without. After adjusting for multiple testing, there were four species associated with higher vaginal glycogen levels: *Streptococcus agalactiae* ($p < 0.001$), *Porphyromonas bennonis* ($p < 0.001$), *Anaerococcus vaginalis* ($p = 0.001$) and *Campylobacter* ($p = 0.001$).

Discussion

The association between vaginal lactobacilli and vaginal health is a core concept in gynecology, but whether lactobacilli are simply a marker of vaginal health or have a functional role in promoting vaginal health is still an active scientific question. The classic description of the postmenopausal vagina is of an environment devoid of lactobacilli, with an elevated pH, although numerous studies show that a significant proportion of women maintain vaginal colonization with lactobacilli after menopause.[4–6] Between 40–80% of women develop vulvovaginal symptoms in menopause,[1,2,22] which can be a source of significant distress.[23] The etiology of these common symptoms is not well understood, which limits treatment options. Identifying whether the vaginal microbiota play a role in genitourinary symptoms of menopause would be an important contribution to our understanding of genital tract pathophysiology.

Little is known about the correlation between report of genitourinary symptoms and the vaginal microbiota. Previous studies correlating the vaginal microbiota with genitourinary

atrophy have relied primarily on composite severity scores based on physical examination findings.[3,4] However, patients' distress derives more from symptoms than an atrophic vulvovaginal appearance. Many studies have attempted to correlate vulvovaginal symptoms with findings of atrophy on exam, with variable success.[3,24,25] We queried women about the presence and severity of vulvovaginal symptoms in some detail. In contrast to other DNA-sequencing-based studies showing lower prevalence of *Lactobacillus* species in women with exam findings of atrophy,[3,4] we did not find any correlations between reported menopausal genitourinary symptoms and the vaginal microbiota. In a study of 59 postmenopausal Chinese women, those who reported symptoms of genitourinary discomfort and had exam findings of atrophy (n=30) had lower proportions of *Lactobacillus* and higher proportions of *Gardnerella* and *Atopobium* than women with no signs or symptoms of atrophy.[6] Most symptomatic women treated with oral estrogen showed an improvement in symptoms and a simultaneous increase in proportion of *Lactobacillus* in the vaginal community. However, no data were presented on whether the 6 women who did not have an increase in lactobacilli showed improvement in symptoms.

We did find an association between vulvovaginal itching and pain and the presence of BVAB1, a Gram negative curved rod highly associated with bacterial vaginosis[26] and pelvic inflammatory disease.[27] Morphologically, this species is similar to *Mobiluncus* and in fact many communities previously identified by microscopy as having *Mobiluncus* may actually have been colonized by BVAB1.[28] In addition to its association with bacterial vaginosis in general, BVAB1 was closely correlated with the presence of a positive "whiff" test in women with bacterial vaginosis, indicating the presence of volatile amines.[19] This bacterium has not yet been cultivated, thus we have limited information about its metabolic capacity and potential for producing compounds that may cause irritation.[29]

Several culture or Gram stain-based studies of the vaginal microbiota have shown decreased prevalence of lactobacilli in the vaginal microbiota in postmenopausal women not on hormone therapy (HT) as compared to premenopausal women.[5,30,31] Recent studies using molecular methods to assess the vaginal microbiota have confirmed these findings[3,4,32] and demonstrated increased diversity of the vaginal microbial community in menopausal women not on HT. While it has long been assumed that these associations are due to a direct correlation between serum estrogen, vaginal glycogen and quantity of lactobacilli, few studies have concretely demonstrated those links. Mirmonsef et al showed a correlation between quantity of vaginal lactobacilli and free vaginal glycogen, but did not see a link between serum estrogen and either vaginal glycogen or *Lactobacillus* levels.[7] In Chinese women with and without vaginal signs and symptoms of atrophy, while serum estradiol levels were similar, relative proportion of vaginal lactobacilli was not.[6] These data, and our findings, suggest that there may not be direct links between serum estrogen, glycogen and *Lactobacillus* colonization as previously postulated.

Estrogen is present in blood in several forms such as estradiol, estrone and estriol which can be free or conjugated via sulfation or glucoronidation.[21] We measured both estradiol and estrone, in both conjugated and unconjugated forms, giving a more comprehensive picture of estrogen levels when compared with previous studies. We found that unconjugated serum estrone was associated with a *Lactobacillus*-dominant microbial community, though not with

any particular *Lactobacillus* species or with quantity of *L. crispatus* or *L. iners.* One possible explanation for the tenuous association between serum estrogen and vaginal lactobacilli is that the ability of the vaginal tissue to respond to estrogen may be more important than simply its presence in determining the local environment. Polymorphisms in the gene for estrogen receptor alpha (ERα) are associated with increased prevalence of vaginal dryness symptoms and hot flashes in postmenopausal women.[33] There are few studies of estrogen receptor phenotype and microbiota, but in mice negative for the ERβ gene the gastrointestinal microbiota are significantly different from mice with the gene present; mice without ERβ were less likely to have *Lactobacilliales* detected in the gastrointestinal microbial community.[34]

While our study focused on the associations between vulvovaginal symptoms, serum estrogen and the microbiota, our population was selected from a hot flash intervention trial, thus the prevalence and severity of vulvovaginal symptoms was relatively low. This limits the generalizability of our results and means that this pilot study had limited power to detect subtle differences in our outcomes of interest. Because the parent study was not focused on vaginal health, we do not have physical exam findings, pH or VMI data to correlate with our measures of symptoms or the microbiota. However, this descriptive analysis is one of the first to evaluate associations between presence and severity of genitourinary symptoms with the vaginal microbiota.

Conclusion

Genitourinary symptoms of menopause are a source of distress for many postmenopausal women. Understanding the pathophysiology of genitourinary symptoms will help identify new strategies for treatment and prevention of this distressing condition. Vaginal colonization with *Lactobacillus* is an important area of focus, but our findings suggest that this may be a marker of vulvovaginal health rather than the agent actively promoting a healthy vagina.

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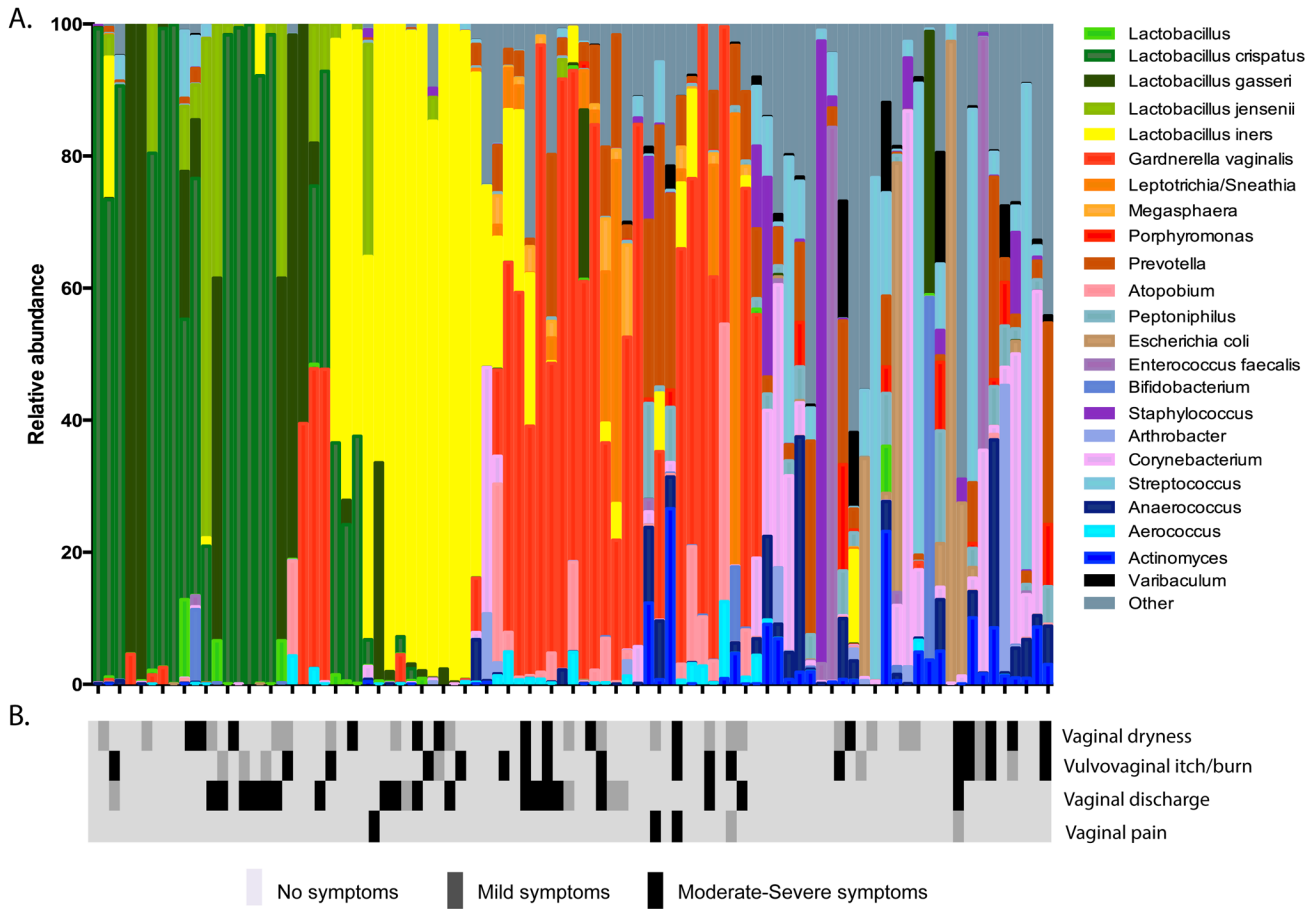

Figure 1.

A. Relative abundance of the most common vaginal bacteria among 88 post-menopausal women, as determined by 16S rRNA gene sequencing and B. presence and severity of patient-reported vulvovaginal symptoms.

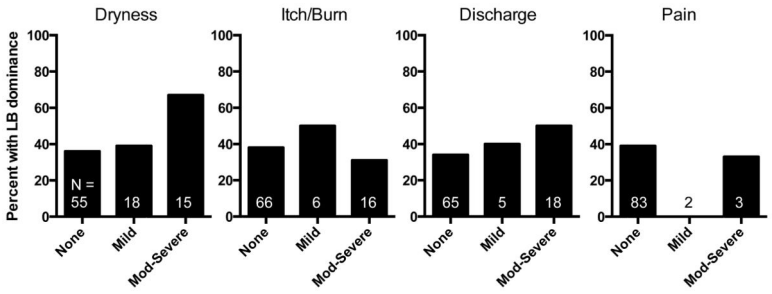

Figure 2.

Proportion of women with *Lactobacillus*-dominant vaginal communities (more than 50% of sequences from *Lactobacillus spp.*) with No, Mild or Moderate-Severe symptoms. Numbers in the bars are the number of participants reporting that severity of symptom. No significant differences were noted in *Lactobacillus*-dominance according to symptom severity for any of the symptoms.

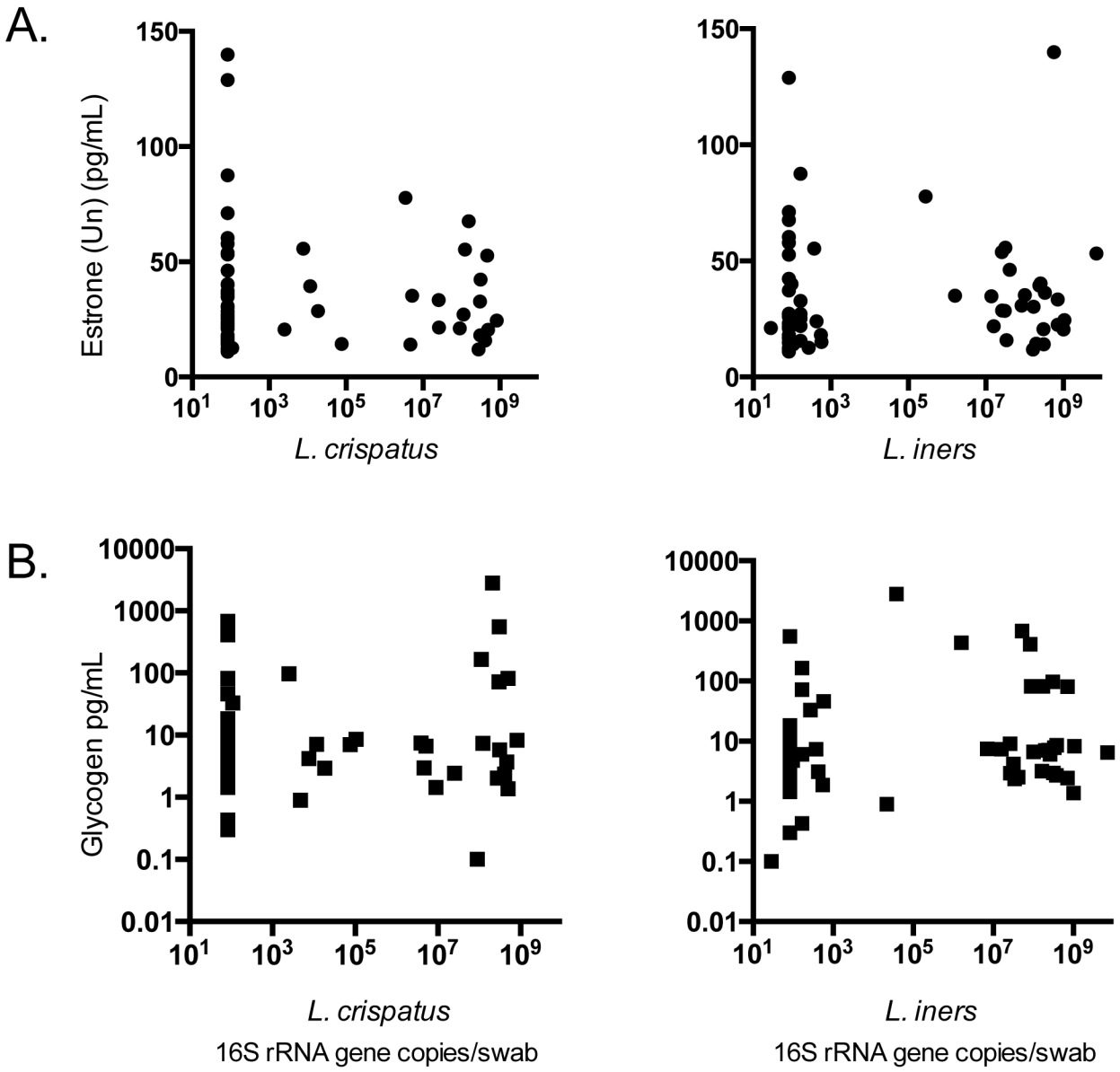

Figure 3.

Correlation between serum levels of unconjugated estrone (A) and vaginal glycogen (B) (Y-axes) and quantity of *L. crispatus* and *L. iners* (X-axis) as measured by 16S rRNA gene qPCR. No significant correlations were noted. The lower limit of detection for the lactobacilli was 83.3 16S rRNA gene copies/swab.

Table 1

Comparison of participant demographic characteristics, serum estrogen, vaginal glycogen and most bothersome genitourinary symptoms between women with and without *Lactobacillus* dominance of the vaginal microbial community (ie. > 50% of sequences from *Lactobacillus* species)

	***Lactobacillus* dominant N = 33**	**Non-*Lactobacillus* dominant N = 55**	**P value**
Demographics			
Age (years; mean ± SD)	53 ± 4	55 ± 4	0.08
BMI (kg/m^2; mean ± SD)	29 ± 5	29 ± 6	0.96
Ethnicity			0.14
White	14 (32%)	34 (62%)	
African American	17 (52%)	17 (31%)	
Hispanic	1 (3%)	2 (4%)	
Asian	0	0	
Other/Mixed	1 (3%)	2 (4%)	
Education			0.60
High school	8 (24%)	10 (18%)	
Vocational/some college	11 (33%)	18 (33%)	
College graduate	7 (21%)	17 (31%)	
Graduate degree	6 (18%)	10 (18%)	
Smoking			0.96
Current	6 (18%)	11 (20%)	
Former	10 (30%)	18 (33%)	
# hot flashes/24h (median, IQR)	6 (5, 8)	7 (4, 11)	0.74
Menopausal status			0.41
Perimenopausal (< 1 yr since LMP)	8 (24%)	9 (16%)	
Postmenopausal (≥ 1yr since LMP)	25 (76%)	46 (84%)	
Years since LMP * (median, IQR)	6 (2, 10)	5.5 (2, 10)	0.83
MENQOL (mean ± SD)	3.6 ± 1.2	3.6 ± 1.3	0.86
GAD-7 (median, IQR)	1 (0, 5)	0.5 (0, 5)	0.77
PHQ-9 (median, IQR)	2 (1, 5)	2 (0, 4)	0.62
Sexually active in past 4 weeks?			
With Men	16 (48%)	27 (49%)	0.93
With Women	0	1 (2%)	-
Self-stimulation	11 (33%)	21 (38%)	0.88

	***Lactobacillus* dominant N = 33**	**Non-*Lactobacillus* dominant N = 55**	**P value**
FSFI (median, IQR)	22 (8, 29)	22 (12, 30)	0.96
Laboratory values			
Vaginal glycogen(pg/mL;median, IQR)	6 (3, 72)	6 (3, 9)	0.66
Serum estradiol (median, IQR)			
Total (pg/mL)	13.5 (0.5, 30.3)	11.4 (4.7, 22.2)	0.99
Unconjugated (pg/mL)	3.8 (0.5, 8.3)	2.6 (0.5, 5.6)	0.40
Serum estrone (median, IQR)			
Total (pg/mL)	198 (139, 363)	181 (117, 293)	0.45
Unconjugated (pg/mL)	33.4 (21.5, 55.3)	22.2 (15, 30.5)	**0.007**
Symptoms			
Most bothersome symptom (MBS)			0.88
Vaginal dryness	8 (24%)	14 (25%)	
Vulvovaginal itch/burn	7 (21%)	8 (15%)	
Vaginal discharge	4 (12%)	5 (9%)	
Pain	2 (6%)	2 (4%)	
Inability to have sex	3 (9%)	9 (16%)	
No bothersome symptom	9 (27%)	17 (31%)	
Severity of MBS (median, IQR)	2 (0, 5)	0.5 (0, 4)	0.24

*Among women reporting ≥ 1 year since last menstrual period (LMP)

MENQOL = Menopause quality of life questionnaire[12]

GAD-7 = Generalized anxiety disorder 7-item questionnaire[14]

PHQ-9 = Patient Health Questionnaire-9[13]

FSFI = Female Sexual Function Index[15]