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ORIGINAL ARTICLE

Lactobacilli and Cytokine Modifications during Menopause and Their Relation to Vulvar and Vulvovaginal Disorders

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Objectives: Female sexual and reproductive health is heavily influenced by the levels and ratios of *Lactobacilli* species and vaginal cytokines. Menopause marks a profound body change as it shifts to a natural and permanent non-reproductive state. Vulvovaginal diseases encompass a broad variety of sexual health conditions. Furthermore, both menopause and vulvovaginal diseases affect vaginal *Lactobacilli* and cytokine levels. Thus, this study aimed to investigate the correlation between menopause, vulvovaginal diseases, and vaginal *Lactobacilli* and cytokine levels.

Methods: Vaginal swab samples were collected as part of a prospective data bank creation to study vaginal conditions as approved by the Institutional Review Board of Texas Tech University Health Sciences Center, Lubbock, USA. This study utilized 38 samples in this database, which were assigned to the pre-menopausal with no vulvovaginal conditions (n = 20) and post-menopausal with vulvovaginal conditions (n = 18) groups. A real-time polymerase chain reaction was conducted to determine the relative concentration of *Lactobacilli* species, while cytokine analysis was performed using multiplex enzyme-linked immunosorbent assay immunoassay. The standardized mean difference, multivariate analysis of variance, and permutational unequal variance *t* test were used for the statistical analysis.

Results: Cytokines, interleukin (IL)-6, macrophage inflammatory protein-1α, IL-8, and *Lactobacillus iners* expression were significantly elevated in the control group compared to the study group ($P = 0.03$ for the cytokines, $P = 0.0194$ for *Lactobacilli*).

Conclusions: The levels of vaginal cytokine and *Lactobacillus* profile were significantly different between the pre-menopausal and post-menopausal groups.

Key Words: Cytokines, *Lactobacillus*, Menopause, Microbiota, Vulvovaginal diseases

INTRODUCTION

The specific microbial communities colonizing the vagina have a profound impact on reproductive and sexual health [1]. Women enter menopause as they leave their reproductive years behind with symptoms including vaginal dryness, fatigue, joint pain, and insomnia [2]. In addition, modifications in *Lactobacilli* and cytokine levels are seen in this period that negatively affects the quality of a woman's life. Vulvovaginal diseases refer to a wide spectrum of health conditions that impact the vulva and vagina. Vulvar and vulvovaginal disorders include lichen sclerosis, bacterial vaginosis, vulvodynia, psoriasis, vestibulectomy, leukorrhea, and candidiasis. Anogenital itching and pain are the most common symptoms attributed to inflammatory dermatoses, infections, neoplasms, hormonal changes, and neuropathies [3,4]. It is vital to provide the clinicians with additional tools/information towards enabling appropriate diagnosis and subsequent treatment.

Lactobacilli are known to be the keystone species of vaginal communities during the reproductive years.

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Several antimicrobial substances including lactic acid, narrow-ranging bacteriocins, and wide-ranging hydrogen peroxide produced by *Lactobacilli* constitute much of the rationale behind their importance within the healthy human vaginal microbiome [5]. Abnormal *Lactobacilli* levels correlate with poor vaginal health and susceptibility to several infections. Low *Lactobacilli* growth is observed in the post-menopausal vagina. This is thought to be influenced by the levels of free glycogen in the vagina, which *Lactobacilli* consume. Relative to pre-menopausal, post-menopausal vagina has a decreased level of available glycogen. The levels of *Lactobacilli* in the vagina, specifically *Lactobacillus iners* and *Lactobacillus jensenii*, are decreased. *L. iners*, *L. jensenii*, *Lactobacillus gasseri*, and *Lactobacillus crispatus* were analyzed for statistical significance between pre-menopausal and post-menopausal women, with results finding *L. iners* to be higher in pre-menopausal samples and *L. jensenii* and *L. crispatus* holding no significant difference [2]. Compare to other defense mechanisms, vaginal *Lactobacilli* such as *L. crispatus*, *L. gasseri*, or *L. jensenii* have the advantage of constraining infection without mounting an inflammatory immune response.

Vaginal cytokines play an important role in regulating the vaginal microbiome as part of the immune system, and their levels change in response to infection, genital abnormalities, pregnancy, and menopause [6]. These signaling proteins mediate the growth and regulation of blood cells and other cells that help the body's immune and inflammatory response. There are both pro-inflammatory and anti-inflammatory cytokines and they can be further divided by their composition and function into lymphokines, monokines, chemokines, and interleukins.

Lactobacilli species can modulate the secretion of certain cytokines and chemokines in response to infections [7]. Invasive anaerobic vaginal microbiota causes bacterial vaginosis and other infections by secreting lipopolysaccharides, resulting in the release of several cytokines and chemokines in response to infection. These agents produce inflammatory responses in the vagina, which can cause vaginal discomfort [1]. These inflammatory cytokines and chemokines are regulated by vaginal *Lactobacilli* [1]. A literature search shows that to date, vaginal cytokines has not been studied extensively in post-menopausal women with vulvovaginal disorders.

The objectives of our study are to determine the differences in vaginal microbiome and cytokine levels between post-menopausal women with vulvar and vulvovaginal disorders and pre-menopausal women without vulvar and vulvovaginal disorders.

MATERIALS AND METHODS

Vaginal swab samples were collected as part of the prospective data bank creation to study vaginal conditions (https://www.clinicaltrials.gov/ct2/show/NCT01829204) as approved by the Institutional Review Board (IRB) of the Texas Tech University Health Sciences Center, Lubbock, TX, USA (IRB No. L21-096). The written informed consent was obtained from all patients enrolled. The age of the patients ranged from 21 to 70 years old. Subjects were divided into two groups: pre-menopausal, control group without any vulvovaginal diseases and post-menopausal with vulvovaginal diseases. Vaginal samples were obtained from the middle of the vagina using standardized cotton swabs [8,9]. Vaginal specimens were added into 1 mL of physiological solution (phosphate-buffered saline) and stored at –80°C at the TTUHSC Permian Basin research lab.

Real–time quantitative polymerase chain reaction (qPCR)

The relative concentration of the vaginal flora was determined by a real-time PCR (qPCR), as described previously [10,11]. The qPCR assay identified vaginal *Lactobacillus* spp., including *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*. In addition, the presence of facultative anaerobic bacteria (*Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma hominis*, and *Mycoplasma genitalium*) were also determined [10]. qPCR analysis was performed. qPCR data were analyzed using the comparative $^{\Delta\Delta}$Ct method [10].

Cytokine evaluation

Cytokine analysis was performed using the Bio-Plex MAGPIX multiplex reader instrument (Bio-Rad, Hercules, CA, USA) and the MesoQuick Plex SQ 120 instrument (Meso Scale Discovery [MSD], Rockville, MD, USA).

1) The Bio-Plex Pro Human cytokine 27-Plex Immunoassay is a 96-well kit that includes all necessary items for the analysis (Cat# M500KCAF0Y; Bio-Rad). The cytokine levels were analyzed according to our previous study [10,11].

2) Cytokine assays through MSD technology provides a rapid and convenient method for measuring cytokine levels. All vaginal swab samples were analyzed using the MSD multiplex instrument MESO QuickPlex SQ 120 (MSD) [12]. The whole procedure of analysis for cytokine expressions was explained in our previous study [10].

Statistical analysis

The project is a retrospective, observational, and cross-sectional study that uses convenience sampling. The study is intended to evaluate the plausibility of significant differences so that no adjustments to *P* values for multiple comparisons are made. A total of twenty control group and eighteen study group patients were used for analyses. Continuous variables are summarized using the mean and SD. Categorical variables are summarized using counts and percentages. For both continuous and categorical variables, effected sizes are reported using the standardized mean difference (SMD). To obtain a single *P* value testing the difference of cytokine concentrations between the groups, parametric bootstrap MANOVA (multivariate ANOVA), defined by Konietschke et al. [13], was applied. This method is appropriate for unbalanced designs, non-normal data, heterogeneity of within group covariance matrices, and allows for computing a single *P* value using all cytokine concentrations. Differences in individual cytokine expressions were analyzed using the permutational Welch unequal variance *t* test.

Within group associations of cytokine concentrations are visualized with heat maps for the Spearman correlation matrix. Each heat map represents 231 different correlations. The study group association matrix is seen to have more, and stronger positive associations compared to the control group association matrix. To obtain a single *P* value measuring the statistical significance of these patterns, the test statistic was chosen as the Frobenius norm of the difference of sample association matrices like the S-statistic used in [14].

$$\|S_{\text{study}} - S_{\text{control}}\|_F = \sqrt{\text{tr}\left[\left(S_{study} - S_{control}\right)^T \left(S_{study} - S_{control}\right)\right]}$$

This test statistic is monotone increasing in the directions toward the alternative hypothesis and has been previously used to the test hypothesis of equal correlation matrices. The *P* value was computed based on

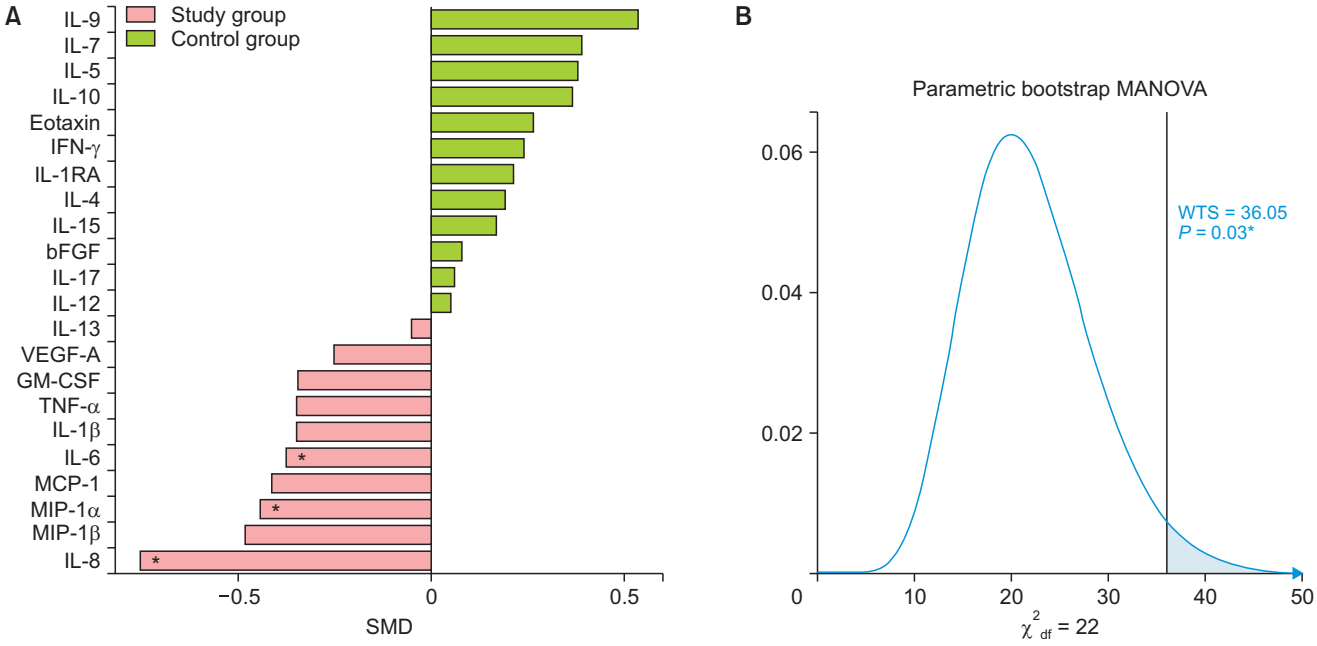

Fig. 1. Standardized difference (SMD) (A) and bootstrap MANOVA (B) for cytokine concentrations. There was a significant difference in cytokine expressions between the control group and the study group ($P = 0.03$). IL: interleukin, IFN: interferon, IL-1RA: IL-1 receptor antagonist, bFGF: basic fibroblast growth factor, VEGF: vascular endothelial growth factor, GM-CSF: granulocyte macrophagecolony stimulating factor, TNF-α: tumor necrosis factor-α, MCP: monocyte chemoattractant protein, MIP: macrophage inflammatory protein, MANOVA: multivariate analysis of variance, df: degree of freedom, WTS: Wald type statistic. *$P < 0.05$.

10,000 permutation distribution null statistics.

Differences in *Lactobacillus* species dominance between patient groups were evaluated using Fisher's test. Within group differences of *Lactobacillus* species dominance was analyzed using Cochran's test. The prevalence of post-menopause vaginal conditions was visualized with a Pareto chart.

Statistical analyses were completed using R version 4.1.1 (http://www.cran.r-project.org), RStudio version 1.4.1717 (http://www.rstudio.com), a significance level of $\alpha = 0.05$, and two-sided *P* values. Power analyses of Fisher's test, the correlation test, and the *t* test are provided in Supplementary Figure 1 (available online).

RESULTS

Patients' characteristics in the control and study groups

Our research study included a total of 20 subjects in the control group and 18 subjects in the study group, all of which reside in the West Texas region, USA. The mean age of the control group was 31.85 years while 58.28 years in the study group (Supplementary Table 1, available online). None of the subjects from the control and study groups had diabetes type 1 comorbid condition. Diabetes type 2 comorbid condition was observed in 27.8% of the subjects in the study group while only 5.0% of the subjects in the control group. Obesity was detected in 60% of the control group while 27.8% in

Table 1. Standardized mean difference (SMD) for cytokine concentrations

Cytokine	Control group (pg/mL)	Study group (pg/mL)	SMD	*P* value
IL-8	348.895	51.497	–0.754	0.009**
IL-6	1.449	0.104	–0.374	0.040*
MIP-1α	4.931	0.793	–0.444	0.047*
IL-9	1.874	4.714	0.536	0.110
MIP-1β	19.317	9.091	–0.482	0.132
MCP-1	15.971	7.951	–0.413	0.216
IL-7	3.166	5.943	0.391	0.242
IL-5	0.012	0.027	0.381	0.255
TNF-α	2.339	0.926	–0.347	0.260
IL-10	1.985	3.001	0.367	0.266
IL-1β	41.813	16.574	–0.348	0.277
GM-CSF	2.920	1.658	–0.346	0.300
VEGF-A	2,059.118	1,593.216	–0.253	0.449
Eotaxin	3.689	5.008	0.267	0.494
IL-1RA	6,841.561	7,837.939	0.215	0.502
IL-4	0.172	0.229	0.192	0.565
IL-15	0.063	0.092	0.169	0.664
INF-γ	7.000	11.203	0.241	0.736
bFGF	1.955	2.182	0.081	0.863
IL-13	3.505	3.289	–0.053	0.864
IL-17a	4.016	4.686	0.062	0.865
IL-12	4.750	5.124	0.052	0.871

Identification and measurement of differences in each cytokine concentration were determined using SMD. IL-6, IL-8, and MIP-1α showed significantly higher levels in the control group.

IL: interleukin, MIP: macrophage inflammatory protein, MCP: monocyte chemoattractant protein, TNF-α: tumor necrosis factor-α, GM-CSF: granulocyte macrophage-colony stimulating factor, VEGF: vascular endothelial growth factor, IL-1RA: IL-1 receptor antagonist, IFN: interferon, bFGF: basic fibroblast growth factor.

*$P < 0.05$, **$P < 0.01$.

the study group. In the study group, 38.9% of patients received hormonal therapy.

Difference in cytokine expression between two groups

Our study demonstrated a significant difference (P = 0.03) in cytokine expressions between the control and the study groups (Fig. 1). Individual cytokines were significantly different between two groups (Table 1). Cytokines, interleukin (IL)-8 (SMD = –0.754, P = 0.009), IL-6 (SMD = –0.374, P = 0.040), and macrophage inflammatory protein-1α (MIP-1α) (SMD = –0.444, P = 0.047) were significantly lower in the study group as compared to the control group (Table 1).

Difference in association patterns between groups

Figure 2A and 2B displays the Spearman correlation matrices for the control and study group, respectively. The study group correlation matrix is seen to have more positive and stronger associations compared to the control group. A permutational test of this pattern results in a low but insignificant P value (P = 0.0966) (Fig. 2C). A similar, but stronger, observation was made in our recent study [10]. In contrast to the overall patterns of positive associations in the study group, IL-6 and IL-8 are observed to have negative associations with almost all other cytokines.

Difference in *Lactobacillus* species between two groups

The vaginal microbiome, particularly *Lactobacillus* species, presence was evaluated and compared between the control and the study groups. *L. iners* (SMD = 0.6969, P = 0.0789) was significantly more detected in the control group compared to the study group. For the control group, Cochran's test (P = 0.0194) indicates significant differences in relative frequency of *Lactobacillus* species dominance, with *L. iners* being significantly more dominant than *L. gasseri* and *L. jensenii*. For the study group, Cochran's test (P = 0.1762) does not indicate statistically significant differences in the relative frequency for *Lactobacillus* species dominance (Fig. 3).

Prevalence of vaginal conditions in menopausal subjects

Figure 4 reports the observed prevalence of vaginal

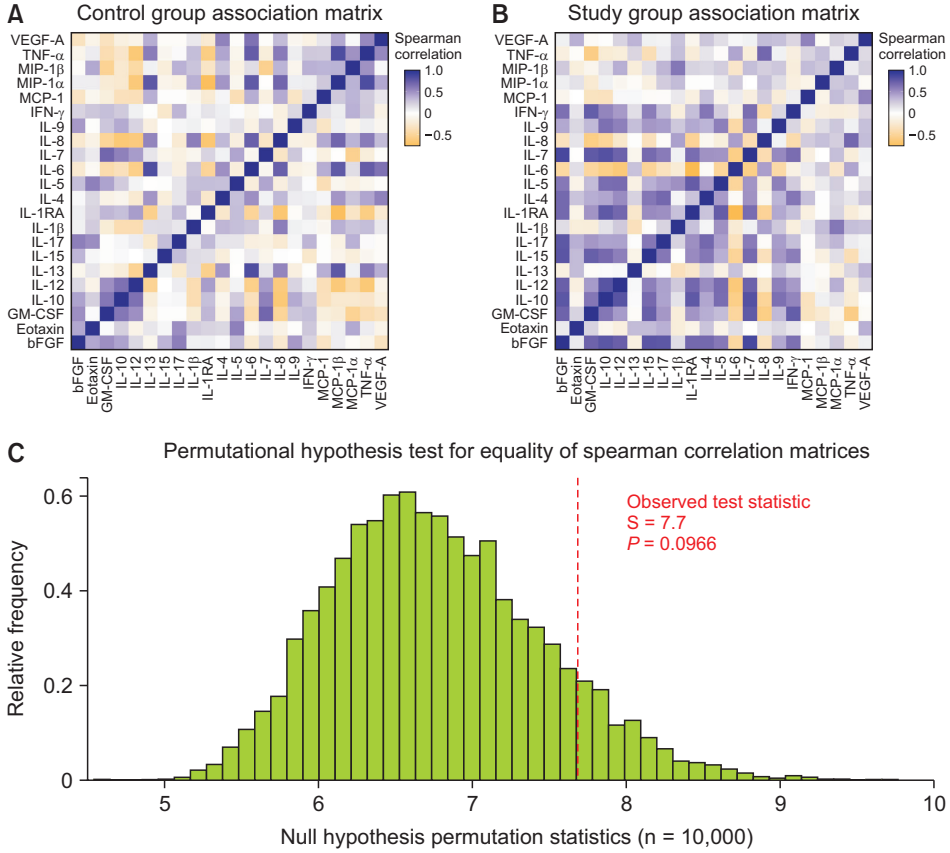

Fig. 2. Association patterns for cytokine concentration in the control (A) and study (B) groups. (C) The correlation matrix in the study group is seen to have more positive and stronger associations compared to the control group. IL-6 and IL-8 are observed to have negative associations with almost all other cytokines. VEGF: vascular endothelial growth factor, TNF-α: tumor necrosis factor-α, MIP: macrophage inflammatory protein, MCP: monocyte chemoattractant protein, IFN: Interferon, IL: interleukin, IL-1RA: IL-1 receptor antagonist, GM-CSF: granulocyte macrophage-colony stimulating factor, bFGF: basic fibroblast growth factor. S: test statistic.

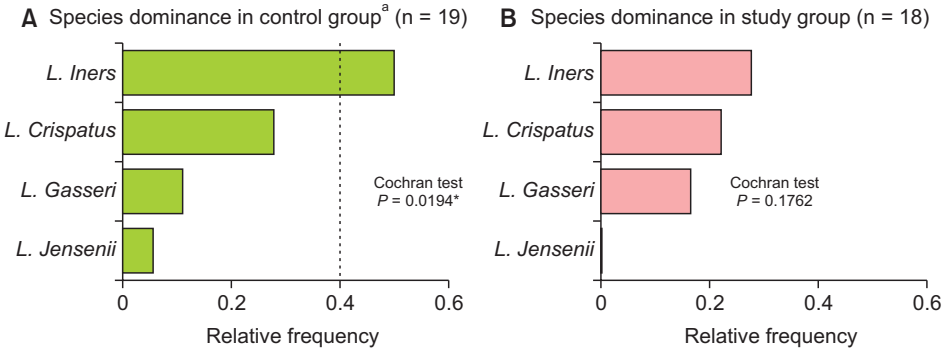

Fig. 3. Patterns of *Lactobacillus* species dominance in the control (A) and study groups (B). *Lactobacillus iners* was expressed significantly higher in the control group. There was no significant difference in *L.* species expression within the study group. *$P < 0.05$. [a]One patient in the control group's data about *Lactobacilli* is missing.

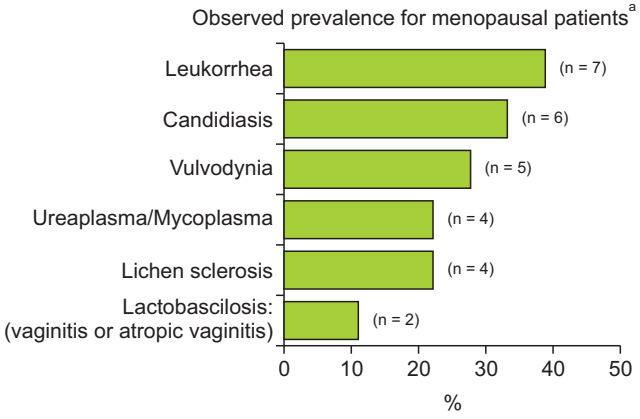

Fig. 4. Observed prevalence of vaginal conditions in the study group (n = 18). Leukorrhea, candidiasis, vulvodynia, ureaplasma/mycoplasma, lichen sclerosis, and lactobacilosis were observed in post-menopausal subjects. [a]Some patients had multiple conditions; this is why the sum in this figure is 28.

conditions in post- menopausal subjects. Leukorrhea was the most prevalent condition (n = 7, 38.9%) and vaginal lactobacillosis (n = 2, 11.1%) was the least prevalent condition (Fig. 4, Supplementary Fig. 1 [available online]).

DISCUSSION

Our research study demonstrated the relative frequencies of *Lactobacilli* species in the control and study groups. *L. iners* was the significantly dominant vaginal *Lactobacilli* species in the control group of research study. When we looked at the prevalence of vaginal conditions in the study group, we noticed leukorrhea was the most prevalent condition and vaginal lactobacillosis was the least prevalent condition. The levels of IL-8, IL-6, and MIP-1α were significantly lower in the study group.

Menopause is associated with dysregulation and a decrease in vaginal immune responses and altered vaginal microbiome, both of which contribute to an increased susceptibility to vaginal infections. In our study, we observed the prevalence of vaginal conditions in the study group and found that leukorrhea was the most prevalent condition, with vaginal lactobacillosis being the least prevalent. Human-based data which studied the association between vulvovaginal disorders, vaginal microbiome, and vaginal immune response are sparse, with the majority of the study focused on natural immunity response and animal models [6]. Furthermore, there is limited data available on modifications of the vaginal microbiome and vaginal cytokine expression in post-menopausal women compared to pre-menopausal women in vaginal swab samples within the West Texas population, USA. The changes in the vaginal microbiome are due to aging and advancement in to menopause also change the vaginal immunity, thus potentially altering the expression of cytokines within the vaginal milieu [6]. With this in mind, our study sought to delineate any cytokine modifications between the study and control groups.

Lactobacilli dominate a healthy human vaginal microbiome and have long been regarded as the keystone microorganism within the human vagina. *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* present as the most abundant species in the vagina. Considered to be beneficial due to their anti-microbial properties, *Lactobacilli* prevent infection due to their production of hydrogen peroxide (H_2O_2), organic acids, and various other anti-infective substances. *L. iners* has the characteristic of staying at relatively low growth in a healthy human vagina but thrive when infection occurs [15]. When comparing the relative species dominance of *Lactobacilli* in the vaginal flora between the pre- and post-menopausal groups in our study, *L. iners* was found to be significantly more dominant relative to other

Lactobacilli species in the control group. The relative dominance of *L. iners* in the control group may be due to factors such as age, and the West Texas location [10]. Of these factors, age plays a crucial role in determining the composition of the vaginal flora. This is especially important when women enter menopause.

In our study, we evaluated the cytokine levels in vaginal swab samples and compared them between our study and control groups. IL-8, IL-6, and MIP-1α were found to be significantly lower in the post-menopausal study group. These three cytokines are pro-inflammatory cytokines and are produced by macrophages and epithelial cells. IL-6 in particular controls the differentiation of monocytes in to macrophages by regulating the expression of macrophage colony-stimulating factor [16]. The innate immune system is highly influenced by sex hormones, and the mechanisms regulated by estradiol levels and their relationship is complex and not fully understood. While lower estradiol levels during menopause generally promote a pro-inflammatory response through altering estrogen receptor activity, it also inhibits pro-inflammatory cytokines under certain circumstances through several mechanisms [17].

Our results are similar to a previous study which demonstrated that IL-6 and IL-8 were at significantly lower levels in the cervical mucosa of post-menopausal women [6]. IL-8 is a chemokine produced by a variety of cells including macrophages, epithelial cells, hepatocytes, and vascular endothelial cells, and is involved in neutrophil recruitment [6,18]. Our results also agree with a previous study which showed the percentage of T cells producing IL-8 decreases with age [19]. While there have been studies conducted on the role of MIP-1α in the vagina with vulvar conditions, there is little work describing the role of MIP-1α in the menopausal cervico-vaginal canal. For example, the earlier study of post-menopausal women demonstrated no difference in MIP-1α levels for vulvo-vaginal irritation. The interplay of these complex immune responses and their interactions with the vaginal micro flora all contribute to sustaining protection against pathogens.

Invasive bacteria in disease conditions in menopausal women stimulate the production of pro-inflammatory IL-6 and IL-8. The primary source of lactic acid in the cervico-vaginal canal is bacteria, not epithelial cells [20]. *L. crispatus* reduces pH to a greater extent than *L. iners* in the cervico-vaginal canal [1,21]. Also, *L. crispatus* produces both D- and L-lactic acid, while *L. iners* produces only L-lactic acid [1,22]. Since our results show that the ratio of *L. crispatus* to *L. iners* is increased in post-menopausal women, it is possible that the post-menopausal women in our study had decreased vaginal pH due to increased secretion of lactic acid and/or changes in the ratio of D and L isomers of lactic acid. The two isomers of lactic acid were shown to have different effects on host gene expression and immune properties [1]. In addition, a decrease in vaginal pH may decrease the ability of the invasive bacteria to stimulate IL-6/IL-8 production. These factors may explain reduced levels of IL-6/IL-8 observed in our study. It is worth noting that lactic acid was shown to inhibit the production of pro-inflammatory cytokine, IL-6 and IL-8, from human vaginal and cervical epithelial cell lines [23]. Further, it was shown that *L. crispatus* secretions decrease the production of IL-6 and IL-8 in ecto-cervical and endo-cervical cells [24]. Aside from the cytokines mentioned previously, our study did not show any significant differences on the other cytokines we evaluated when comparing them between our control and study groups. Although the methodology used in this study is very sensitive, we believe that this result is mainly due to a small sample size in the control and study groups. As such, future work with a sufficiently larger sample size is needed to further study the correlation between menopause, vaginal pH, levels of D- and L-lactic acid isomers, predominance of *Lactobacilli* species and its influence on vaginal cytokines and overall vaginal immunity.

In conclusion, our study demonstrated a significant difference ($P = 0.0194$) in relative *Lactobacilli* species dominance when compared to our control and study group. *L. iners* was found to be the dominant *Lactobacilli* species present in post-menopausal women. We also evaluated several cytokines from vaginal swab samples and demonstrated a significant difference in cytokine expressions between the control and the study groups. Pro-inflammatory cytokines IL-8 ($P = 0.009$), IL-6 ($P = 0.040$), and MIP-1α ($P = 0.047$) were significantly reduced in the post-menopausal group and we anticipate the observed differences in the vaginal microbiome and cytokine levels between the pre- and post-menopausal groups to be due to changes in lactic acid production.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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