



Composition of the vaginal microbiota in relation to cervical intraepithelial lesions

Servikal intraepitelyal lezyonlarla ilişkili vajinal mikrobiyota kompozisyonu

İ Ethem Serdar Yalvaç¹, İ Çağlayan Ateş¹, İ Mustafa Bakırcı¹, İ Emine Yeşilyurt Şölen²,
İ Fulya Kayıkçıoğlu³, İ Sevgi Koç³, İ Ayşe Erbay⁴, İ Demet Aydoğan Kırmızı¹, İ Taylan Onat¹

¹Yozgat Bozok University Faculty of Medicine, Department of Obstetrics and Gynecology, Yozgat, Türkiye

²Yozgat Bozok University Faculty of Medicine, Department of Microbiology, Yozgat, Türkiye

³University of Health Sciences Türkiye, Etlik Zübeyde Hanım Training and Research Hospital, Department of Obstetrics and Gynecology, Ankara, Türkiye

⁴Yozgat Bozok University Faculty of Medicine, Department of Infectious Diseases and Clinical Microbiology, Yozgat, Türkiye

Abstract

Objective: This study aimed to investigate the relationship between cervical intraepithelial neoplasia associated with human papillomavirus (HPV) infection and the vaginal microbiome.

Materials and Methods: In this study, the vaginal microbiota profile was compared among three groups of women: those with HPV infection and no cervical intraepithelial neoplasia (NILM, n=35), those with low-grade squamous intraepithelial lesions (LSIL, n=28), and those with high-grade squamous intraepithelial lesions (HSIL, n=24). Vaginal bacterial diversity was analyzed by deep sequencing of the V3-4 region of the barcoded 16S rRNA gene using the Illumina MiSeq platform, considering alpha diversity, beta diversity, and taxon classifications. Statistical significance was set at p<0.05.

Results: In the analyses performed using Chao1, Inverse Simpson, Shannon, and Observed indices, statistically significant differences were found among the groups in terms of all indices (p<0.05). Among groups, beta diversity did not show any notable differences. According to the "Linear Discriminant Analysis Effect Size" analysis, the taxa enriched in the HSIL group were *Roseburia inulinivorans* (p=0.0308), *Micromonosporaceae* family (p=0.0331), and *Pirellula* genus and species, (Planctomycetes), (p=0.0165); the taxa enriched in the LSIL group were *Actinobaculum* genus and species (p=0.0183). *Lactobacillus helveticus* and *Faecalibacterium prausnitzii* were more abundant in the NILM group, while *Prevotella copri*, *Akkermansia muciniphila*, and *Fusobacterium* species were more abundant in the LSIL and HSIL groups.

Conclusion: Our findings indicate that variations in the severity of cervical lesions are associated with notable alterations in vaginal microbiota composition. Further research is required to conclude which contribute to the formation of the cervical lesion and which are a consequence, among those that cause changes in the vaginal microbiota, of the lesion.

Keywords: Microbiota, cervical intraepithelial neoplasia, human papillomavirus

Öz

Amaç: Bu çalışmanın amacı insan papilloma virüsü (HPV) enfeksiyonu ile ilişkili servikal intraepitelyal neoplazi ve vajinal mikrobiyom arasındaki ilişkiyi araştırmaktır.

Gereç ve Yöntemler: Bu çalışmada, vajinal mikrobiyota profili üç grup kadın arasında karşılaştırıldı: HPV enfeksiyonu olan ve servikal intraepitelyal neoplazisi olmayanlar (NILM, n=35), düşük dereceli skuamöz intraepitelyal lezyonu olanlar (LSIL, n=28) ve yüksek dereceli skuamöz intraepitelyal lezyonu olanlar (HSIL, n=24). Vajinal bakteri çeşitliliği, Illumina MiSeq platformu kullanılarak barkodlanmış 16S rRNA geninin V3-4 bölgesinin derin sekanslanmasıyla alfa çeşitliliği, beta çeşitliliği ve takson sınıflandırmaları dikkate alınarak analiz edilmiştir. İstatistiksel anlamlılık p<0,05 olarak belirlenmiştir.

PRECIS: Comparison of human papillomavirus genotype distribution and microbiota profiles in women with varying grades of cervical epithelial abnormalities.

Corresponding Author/Sorumlu Yazar: Çağlayan Ateş MD,

Yozgat Bozok University Faculty of Medicine, Department of Obstetrics and Gynecology, Yozgat, Türkiye

E-mail: caglayanctf@hotmail.com ORCID ID: orcid.org/0000-0002-4200-2659

Received/Geliş Tarihi: 02.05.2025 Accepted/Kabul Tarihi: 31.07.2025 Epub: 05.07.2025

Cite this article as: Yalvaç ES, Ateş Ç, Bakırcı M, Yeşilyurt Şölen E, Kayıkçıoğlu F, Koç S, et al. Composition of the vaginal microbiota in relation to cervical intraepithelial lesions. Turk J Obstet Gynecol. [Epub Ahead of Print]



Bulgular: Chao1, Inverse Simpson, Shannon ve Observed indeksleri kullanılarak yapılan analizlerde, tüm indeksler açısından gruplar arasında istatistiksel olarak anlamlı farklılıklar bulunmuştur ($p<0,05$). Beta çeşitliliği açısından gruplar arasında önemli bir farklılık gözlenmemiştir. “Lineer Diskriminant Analizi Etki Büyüklüğü” analizine göre, HSIL grubunda zenginleşen taksonlar: *Roseburia inulinivorans* ($p=0,0308$), *Micromonosporaceae* familyası ($p=0,0331$), *Pirellula* cinsi ve türleri (Planctomycetes) ($p=0,0165$); LSIL grubunda zenginleşen taksonlar: *Actinobaculum* cinsi ve türleri ($p=0,0183$). *Lactobacillus helveticus* ve *Faecalibacterium prausnitzii* NILM grubunda daha bol bulunurken, *Prevotella copri*, *Akkermansia muciniphila* ve *Fusobacterium* türleri LSIL ve HSIL gruplarında daha bol bulunmuştur.

Sonuç: Bulgularımız, farklı derecelerde servikal lezyonları olan kadınların vajinal mikrobiyota profillerinde önemli değişiklikler olduğunu ortaya koymaktadır. Vajinal mikrobiyotadaki bu değişikliklere neden olan biyolojik ajanlardan hangilerinin servikal lezyon oluşumuna katkıda bulunduğu ve hangilerinin lezyonun bir sonucu olduğu sonucuna varmak için daha fazla araştırma yapılması gerekmektedir.

Anahtar Kelimeler: Mikrobiyota, servikal intraepitelyal neoplazi, insan papilloma virüsü

Introduction

Human papilloma virus (HPV) is the most common infection among sexually transmitted infections, and the lifetime infection rate in women who continue their sexual life can reach 70% and above⁽¹⁾. Epidemiological data show that most of these infections disappear spontaneously in the short term thanks to the body's own immune mechanisms. However, in 10% to 20% of cases, the infection becomes persistent, and a long-term clinical course is observed^(1,2). Persistent infections predispose the development of cervical squamous intraepithelial lesions (SIL), which can develop into low-grade squamous intraepithelial lesions (LSIL) or high-grade intraepithelial lesions (HSIL). HSIL carries a high risk of carcinogenesis and requires early intervention against the risk of viral DNA integration into the cell genome. In contrast, LSIL cases are usually associated with transient HPV infections, and approximately 60% of cases tend to resolve naturally within one year⁽³⁾.

The vaginal microbiome has long been identified as playing a critical role in maintaining vaginal health. Next-generation sequencing technologies provide more detailed and in-depth information about the structure and functions of bacterial communities in the vagina^(4,5). Dysbiosis in the vagina causes damage at the cellular and DNA level by producing bacterial genotoxins, thus triggering the formation of a chronic inflammatory environment favorable for cancer development⁽⁶⁾. Chronic inflammation triggers the production of nitrosamines, paving the way for metabolomic profiles considered to be associated with cancer to play a decisive role in the interaction between cervical inflammation, HPV infection, and the local microbiome^(7,8).

Previous studies have revealed that *Lactobacillus* dominance decreased and bacterial diversity increased in the vaginal microbiome of women diagnosed with HPV persistence, LSIL, and HSIL^(9,10). In addition, although specific bacterial species associated with HPV and SIL have been identified, no consensus has been reached on this issue⁽¹¹⁾. Therefore, in this study, only the differences between vaginal dysbiosis and the normal microbiome of women diagnosed with LSIL and HSIL were analysed.

Materials and Methods

Ethical Approval

This is a prospective multi-center study of adult women admitted to the colposcopy units of Bozok University Research and Application Hospital and Etlik Zübeyde Hanım Gynecology Training and Research Hospital. Participants were enrolled between January 2023 and January 2024. The research protocol received ethical clearance from the Clinical and Interventional Research Ethics Committee of Yozgat Bozok University Research and Application Hospital, with official approvals granted on 25 August 2022 and 22 September 2022 (decision no: 2017-KAEK-189_2022.08.25_10, date: 28.08.2022 and decision no: 2017-KAEK-189_2022.09.22_06, date: 22.09.2022). All authors fully complied with the ethical principles of the Declaration of Helsinki.

Study Design and Sample Collection

Pre-menopausal, non-pregnant women over the age of 30 years who presented to the Clinic of Colposcopy, Yozgat Bozok University Research and Application Hospital and Etlik Zübeyde Hanım Training and Research Hospital, with a diagnosis of HPV positivity, were included in the study. In this study, we excluded individuals who met the following criteria: 1) those who had received antibiotic treatment in the last 7 days, 2) those who had used suppositories, vaginal medication or vaginal cleaning products in the 48 hours before the visit, 3) those who had a history of coitus within the last 48 hours, 4) those currently diagnosed with a sexually transmitted infection or vaginal infection, 5) those with diseases affecting the immune system, 6) those who had undergone cervical treatment in the past, 7) those using hormone replacement therapy or oral contraceptives, and 8) active smokers. All participants provided written informed consent.

A total of 87 women who participated in the study were divided into three groups according to their cervical pathological findings based on the Bethesda 2001 system⁽¹²⁾ from the histopathological evaluation of the biopsy samples taken under colposcopy: No intraepithelial lesion or malignancy (NILM) ($n=35$), LSIL ($n=28$), and HSIL ($n=24$). The presence of HPV was detected by Linear Array HPV Genotyping Assays developed

by Roche (Indianapolis, IN) and the subjects were divided into three categories as follows: 1) HPV 16 and 18 positive, 2) Other high-risk subtypes positive, and 3) Low-risk subtypes positive (Table S1). Other high-risk HPV types include HPV26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, and 82. The low-risk HPV comprises HPV6, 11, 40, 42, 43, 44, 54, 61, and 70. Some demographic characteristics of all included patients were also recorded. It is shown in Table 1.

Two gynecologists from each of the two centers used eNATTM kits (606CS01L, Copan Group, Copan Italia), to collect vaginal swabs without lubrication after inserting the vaginal speculum. Each vaginal sample was collected using a different swab and collecting tube, and it was promptly stored at -80 °C until DNA extraction.

DNA Isolation

After the samples were collected, they were all sent to the Diagen lab in Ankara, Türkiye, on dry ice so that DNA could be extracted. As directed by the manufacturer, vaginal swab samples were examined using the Kurabo QuickGene DNA tissue kit S (Japan).

DNA Amplification and Sequencing of 16S RNA Gene Fragments

Total genomic DNA was extracted from vaginal samples with the CTAB/SDS technique. DNA concentration and purity were evaluated via electrophoresis on a 1% agarose gel, after which the samples were adjusted to a final concentration of 1 µg/µL using sterile water. The V3-V4 hypervariable portions of the bacterial 16S rRNA gene were subsequently amplified according to the designated polymerase chain reaction (PCR) conditions. In this procedure, the primers 515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3' were utilized. DNA amplification was conducted following the Illumina technique for 16S Metagenomic Sequencing Library Preparation (Part No. 15044223 Rev. A, Illumina, CA, USA). PCR processes

commenced with an initial denaturation at 98 °C for 1 minute, succeeded by 30 cycles including denaturation at 98 °C for 10 seconds, annealing at 50 °C for 30 seconds, and elongation at 72 °C for 30 seconds. PCR products were assessed using 2% agarose gel electrophoresis and subsequently purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). The PCR products were indexed using the Nextera XT Index Kit (Illumina), followed by the multiplexing step. Purified DNA libraries were constructed utilizing TruSeq DNA PCR-free sample preparation kits (Illumina, USA) and sequenced to acquire 250 bp paired-end reads using the Illumina NovaSeq platform. Following sequencing, barcodes were eliminated from the arrays, and the data were analyzed using the QIIME V.2.0 analytical pipeline. The amplicon sequence variations (ASVs) for each sample were limited to 10,000 reads through random selection^(13,14). The ASV taxonomy was established using the Silva 138 SSURef NR99 16S rRNA gene reference database⁽¹⁵⁾. Reliability was maintained throughout the experimental process by employing negative controls in DNA extraction, PCR, and sequencing. The precision of the studies was enhanced by employing an internal standard, which included an internal control for 16S and 1007 reads, during sequencing.

Bioinformatics Analysis of Sequences

First, a quality evaluation was conducted using the Prinseq-lite (v0.20.4) software (<http://prinseq.sourceforge.net>) to begin bioinformatic analysis of the raw read data. The quality control step was conducted using the following parameters: 50 for min length, 30 for trim qual right, 20 for trim qual window, and mean for trim qual type. The FLASH software was used with the default parameters to merge the quality-controlled forward and reverse read sequences. After merging, human-derived sequences were identified by aligning individual reads to the human genome (GRCh38.p11, December 2013) using Bowtie2 software (very-sensitive mode: -D 20 -R 3 -N 0 -L 20 -i S,1,0.50) and subsequently removed.

Table 1. Clinical characteristics of subjects in each group

Characteristics	NILM group (n=35)	LSIL group (n=28)	HSIL group (n=24)	p value
Age (years)	41.44±10.506	41.18±9.851	42.58±9.297	0.955 [#]
Height (m)	1.60±0.739	1.60±0.526	1.61±0.665	0.657 [*]
Weight (kg)	70.85±10.59	69.36±10.302	71.50±7.530	0.696 [*]
BMI	22.11±2.770	21.98±3.254	22.24±3.110	0.420 [*]
Gravida	2.91±1.640	2.46±1.401	3.33±1.810	0.070 [*]
Parity	2.50±1.308	2.91±1.640	2.82±1.416	0.182 [*]
Duration of marriage	19.29±10.823	18.5±12.670	24.17±12.240	0.067 [*]
Number of previous miscarriages	0.35±0.485	0.46±0.508	0.37±0.495	0,65 [#]

Values are mean ± SD for continuous variables. NILM: No intraepithelial lesion or malignancy, LSIL: Low-grade squamous intraepithelial lesions, HSIL: High-grade intraepithelial lesions, BMI: Body mass index, SD: Standard deviation, *: ANOVA, #: Kruskal-Wallis

Using the Bayesian-based ribosomal database project (RDP) Classifier (v2.12) algorithm, reads that did not match the human genome were taxonomically categorized after they were aligned to the RDP. A contingency table with taxonomic counts was created once each sample's taxonomic distribution was established. QIIME (Quantitative Insights Into Microbial Ecology) v1.9.0 software was used to convert this table to Biom format, and the ecological diversity, abundance, and composition of microorganisms were analyzed⁽¹⁶⁾.

The diversity within samples was measured using the alpha diversity assessment. With 9,500 random readings, 1,000 dilutions were made for every sample. The Shannon diversity index, Chao1 index, observed index, and inverse Simpson index were used per sample to determine diversity. The statistical software R (v3.1.0) was used to create box plots to visualize the results of the alpha diversity analysis.

The microbiological diversity of the samples was examined using beta diversity assessments. To do this, Bray-Curtis distance index matrices were used in principal coordinate analysis. Utilizing Adonis nonparametric analysis of variance, statistical differences across groups were assessed. Additionally, the non-parametric Wilcoxon test was used in R scripts to analyze groups and sample types.

Statistical Analysis

Data analysis was performed using IBM SPSS Statistics for Windows (version 27.0; Armonk, NY, USA). For continuous variables, descriptive statistics such as mean, standard deviation, and median were calculated. Categorical variables were presented as frequencies and percentages. The Student's t-test was applied to compare numerical data between two groups. For comparisons involving more than two groups, either the Kruskal-Wallis H test or one-way ANOVA was utilized, depending on the normality of the data distribution. A p-value of less than 0.05 was considered statistically significant.

Results

There were no statistically significant differences among the groups in their demographic profiles, including age, height, weight, body mass index, gravida, parity, duration of marriage, or the number of prior abortions ($p > 0.05$) (Table 1).

In the study, analyses of vaginal microbiota alpha diversity between NILM, LSIL, and HSIL groups using Chao1, Inverse Simpson, Shannon, and Observed indices, showed statistically significant differences between the groups in terms of all indices ($p < 0.05$). The Chao1 index (Figure 1A) showed that the LSIL group had significantly higher species richness than the other groups ($p < 0.001$). The Inverse Simpson index (Figure 1B), a measure that emphasizes the distribution of dominant species within the community, indicated higher diversity in the LSIL group; a statistically significant difference was found between the groups ($p < 0.001$). The Shannon index (Figure 1C) was evaluated considering both species richness and even distribution. Alpha diversity analysis revealed significantly

increased richness in the LSIL and HSIL groups relative to the NILM group ($p = 0.009$). As shown in Figure 1D, the LSIL group exhibited the highest observed species count, whereas the NILM group demonstrated the lowest ($p < 0.001$).

Bray-Curtis non-metric multidimensional scaling (NMDS) analyses (Figure 2) revealed that the groups did not differ significantly in terms of beta diversity. No significant difference was observed between the LSIL, HSIL, and NILM (normal) groups. Unweighted UniFrac, and weighted UniFrac NMDS analyses (Figure 2) also showed similar results. There was no significant difference in microbiota composition between the groups. Stress plot analyses showed that the NMDS model was reliable ($R^2 > 0.96$) (Figure 3). The high model fit indicates that the analyses were technically correct, but there was no significant segregation between the groups (Figures 2 and 3).

According to linear discriminant analysis effect size (LEfSe) analysis used to determine the enriched microbiota species specific to the groups, the enriched taxa in the HSIL group were *Roseburia inulinivorans* ($p = 0.0308$, LDA score = 2.50), *Micromonosporaceae* family ($p = 0.0331$, LDA score = 2.07), *Pirellula* genus and species (*Planctomycetes*) ($p = 0.0165$, LDA score = 2.07); in the LSIL group, the enriched taxa were *Actinobaculum* genus and species ($p = 0.0183$, LDA score = 3.16); and in the NILM group, the enriched taxa were *Deinococci* class ($p = 0.0301$, LDA score = 2.34), *Thermus* genus and *Thermaceae* family ($p = 0.0301$, LDA score = 2.34) (Figure 4 and S1).

In this study, the distribution of vaginal microbiota at phylum, class, order, family, genus, and species levels among NILM, LSIL, and HSIL groups was analyzed.

When analyzed at the phylum level, Firmicutes emerged as the predominant phylum across all groups. In contrast, the relative abundances of *Bacteroidetes* and *Actinobacteria* were reduced in both LSIL and HSIL groups. Meanwhile, phyla like *Fusobacteria* and *Proteobacteria* showed a marked increase, particularly in the HSIL group (Figure 5A).

At the class level, *Bacilli* and *Clostridia* were observed as dominant in all groups. *Gammaproteobacteria* and *Actinobacteria* had higher proportions in LSIL and HSIL groups; and especially *Verrucomicrobiae* and *Synergistia* increased in the lesion group (Figure S2). *Lactobacillales*, the most common bacterial group at the order level, had the highest proportion in all groups. *Bacteroidales* and *Clostridiales* were more prominent in the HSIL and LSIL groups, while *Fusobacteriales* and *Campylobacteriales* were significantly increased, especially in the HSIL group (Figure S2). At the family level, *Lactobacillaceae* was the most common family in all groups. *Prevotellaceae* and *Leptotrichiaceae* were more prevalent in the HSIL group, while *Bifidobacteriaceae* and *Ruminococcaceae* were more prevalent in the LSIL and NILM groups. *Fusobacteriaceae* and *Veillonellaceae* were among the other important families showing an increase in the HSIL group (Figure S2). At the genus level, *Lactobacillus* was the dominant genus in all groups. *Prevotella* and *Gardnerella* were found at higher rates in both the LSIL and HSIL groups, while *Atopobium*

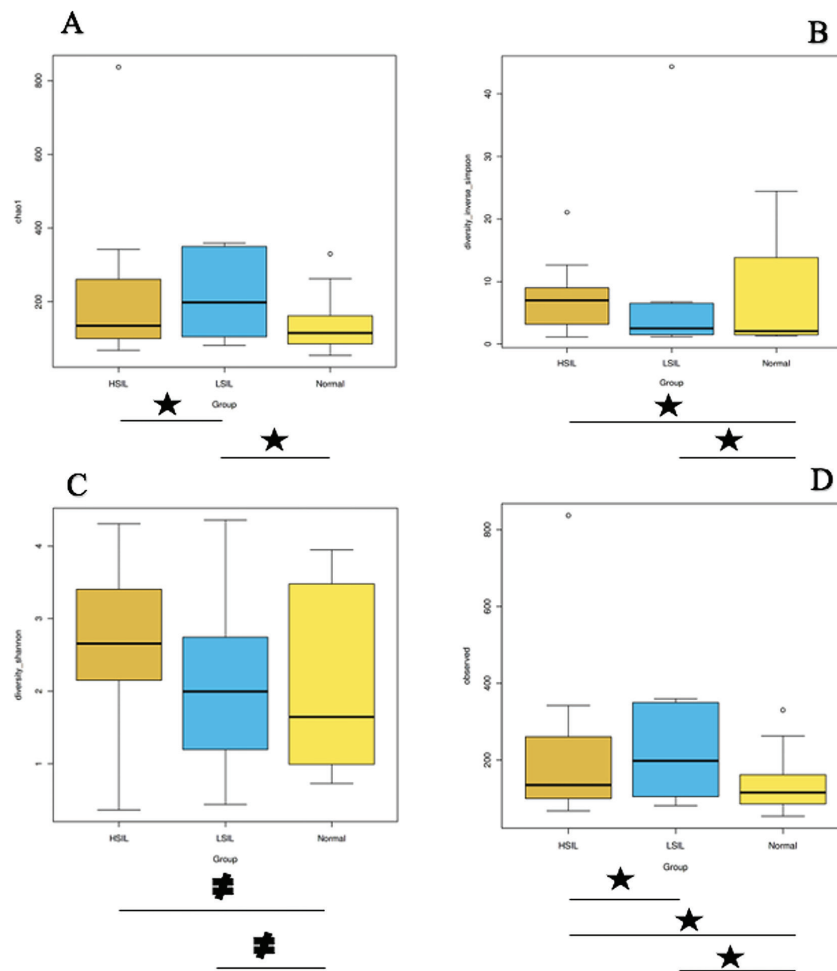


Figure 1. Comparison of groups in terms of alpha diversity. 1A: Species richness assessment with Chao1 index. A significant increase was observed in the LSIL group, 1B: Analysis of dominant species distribution by Inverse Simpson index. The highest diversity was observed in the HSIL group, while a wider distribution was observed in the NILM group, 1C: Analysis of species diversity and even distribution with Shannon index. Higher diversity was found in LSIL and HSIL groups, 1D: Box plot showing differences between groups for number of observed species. Star (*): $p < 0.001$; hash (#) $p = 0.009$

NILM: No intraepithelial lesion or malignancy, LSIL: Low-grade squamous intraepithelial lesions, HSIL: High-grade intraepithelial lesions

and *Fusobacterium* appeared more frequently in the HSIL group. *Streptococcus* and *Bifidobacterium* were more common in the NILM group (Figure S2).

At the species level, *Lactobacillus iners* was the most dominant species in all groups. *Lactobacillus helveticus* and *Faecalibacterium prausnitzii* were more abundant in the NILM group, while *Prevotella copri*, *Akkermansia muciniphila*, and *Fusobacterium* species were more abundant in the LSIL and HSIL groups. Species such as *Roseburia faecis* and *Dorea formicigenerans* also showed higher abundance in the NILM group compared to the other groups (Figure 5B).

Discussion

Maintaining the balance of the cervicovaginal microbiota is essential for modulating the acquisition, persistence, and eventual elimination of HPV from the female reproductive

tract. An imbalance in this microbial ecosystem, referred to as dysbiosis, can contribute to the initiation of pathological processes that may lead to carcinogenesis. These processes are mediated through disruptions such as impairment of epithelial integrity, metabolic dysfunction, irregular cellular proliferation, genomic instability, chronic inflammatory responses, and enhanced angiogenesis⁽¹⁷⁻¹⁹⁾.

The vaginal microbiome is a constantly changing ecosystem that can be influenced by several factors⁽²⁰⁾. Ravel et al.⁽⁵⁾ conducted a study where they categorized the vaginal microbiome of healthy females into four distinct community status types (CST), based on the dominating species of *Lactobacillus* and the corresponding pH values. The four CST types are CST-I, CST-II, CST-III, and CST-V, which correspond to the presence of *Lactobacillus crispatus* (26.2%), *L. gasseri* (6.3%), *L. iners* (34.1%), and *L. jensenii* (5.3%), respectively. In subsequent

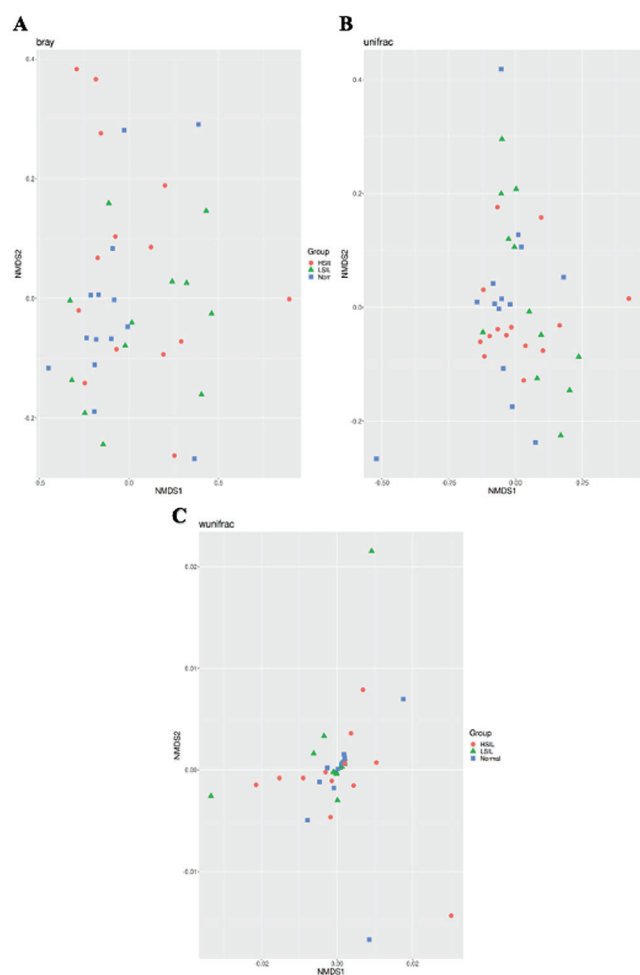


Figure 2. NMDS graphs. 2A: Bray-Curtis NMDS graph. No obvious segregation between groups was observed; 2B: Unweighted UniFrac NMDS plot. No significant segregation was found between the groups; 2C: Weighted UniFrac NMDS plot. Groups were similar in terms of microbiota composition

NMDS: Non-metric multidimensional scaling

years, by assembling the largest dataset of human vaginal microbiota profiles, CST-IV was divided into seven subgroups according to the dominant *non-lactobacillus* species: CST IV-A; *Candidatus lachnocurva vagina* high rate *G. vaginalis* a medium rate, CST IV-B; *G. vaginalis* at a high rate, *L. vaginae* at a low rate, CST IV-C0; an equal community with moderate amounts of *Prevotella*, CST IV-C1; *Streptococcus* dominant, CST IV-C2; *Enterococcus* dominant, CST IV-C3; *Bifidobacterium* dominant, CST IV-C4; and *Staphylococcus* dominant⁽²¹⁾. These members of the flora defend against harmful bacteria by generating hydrogen peroxide (H₂O₂) and bacteriocins, specifically generated by *Lactobacillus* species to maintain an acidic vaginal pH^(22,23). They produce hemolysin to enhance cytotoxicity (*Prevotella*)⁽²⁴⁾ and facilitate adhesion for epithelial colonization (*Atopobium*, *Bifidobacteria*, *Gardnerella*)⁽²⁵⁾.

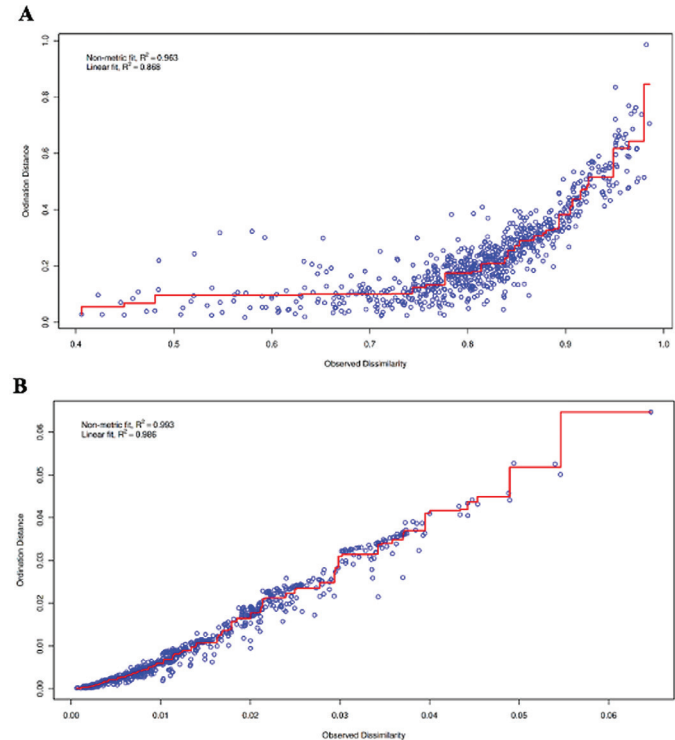


Figure 3. Stress plot analyses. A-B: While showing that the NMDS model is reliable, it supports that there is no significant difference between the groups in terms of beta variation
NMDS: Non-metric multidimensional scaling

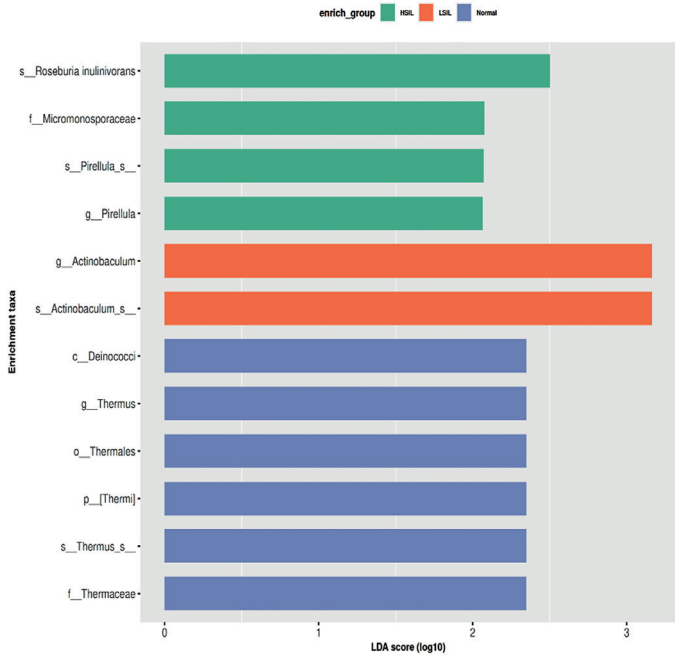


Figure 4. Linear discriminant analysis effect size analysis

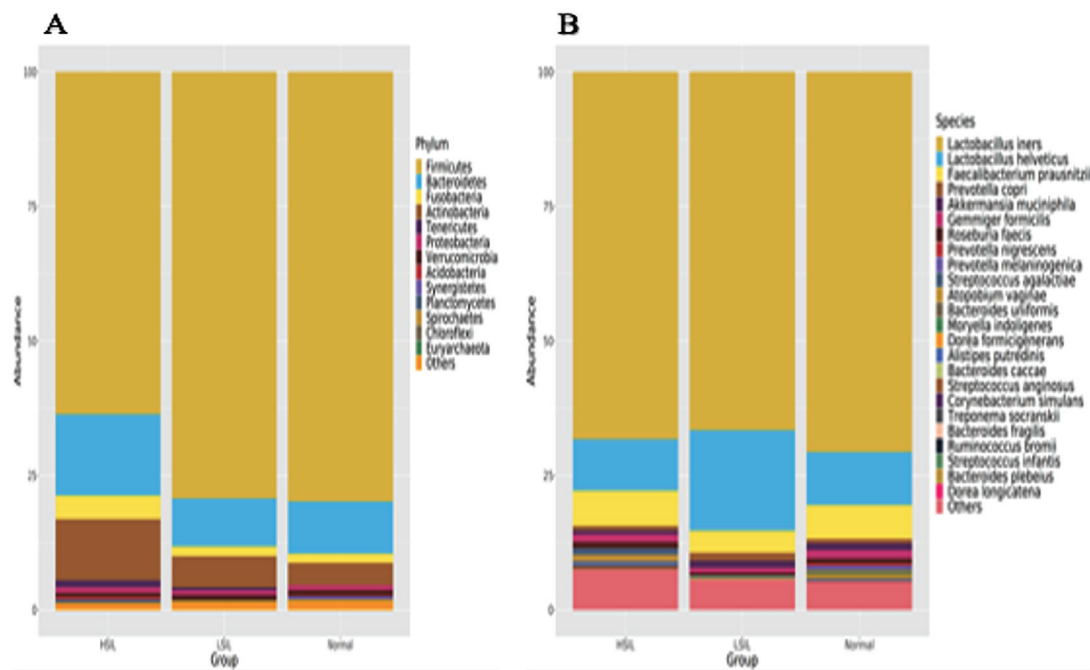


Figure 5. Taxa bar plots A. Bacteria abundance distribution at phylum level, B. Bacteria abundance distribution at species level

CST III and IV have frequently been linked to the emergence of premalignant cervical lesions and the progression to invasive cervical cancer, particularly in the presence of HPV infection. Furthermore, *Lactobacillus gasseri*, the predominant strain of CST II, has been suggested to be associated with the fastest clearance of acute HPV infection in HPV-positive women^(26,27). Some studies report that HPV alters the composition of the vaginal microbiota by shifting it from CST III to CST IV, and that CST IV contributes to the progression and increased severity of cervical neoplasms⁽²⁸⁾. In the present study, *Lactobacillus iners* increased more in the LSIL group than in the HSIL and in the normal group. *Lactobacillus gasseri* was not detected in HSIL and LSIL groups. In accordance with the report by Lee et al.⁽²⁹⁾, which stated that *Prevotella* species are associated with HPV, *Prevotella* species were more prevalent in HSIL groups in our study. *Fusobacteria* were the predominant phylum, especially in the HSIL group. This is consistent with other studies emphasizing the association between *Fusobacteria* and cervical cancer so far^(9,29,30).

LEfSe analysis conducted in our study revealed several candidate biomarkers that were linked to distinct stages of cervical pathology. *Roseburia inulinivorans* and members of the *Micromonosporaceae* family were found to be significantly more abundant in the HSIL group, while *Actinobaculum* was predominantly detected in LSIL samples. *Roseburia inulinivorans* is a motile bacterial species classified under the phylum *Firmicutes*, typically residing in the human colon, where it aids in butyrate synthesis through the fermentation of dietary polysaccharides⁽³¹⁾. On the other hand, *Actinobaculum* is known to be a common pathogen in urogenital tract infections⁽³²⁾.

These two agents are of interest for HSIL and LSIL, considering the exclusion criteria and meticulous sampling.

Akkermansia muciniphila was expressed at higher levels in HSIL and LSIL groups compared to other groups. Considering the reports in previous studies that *Akkermansia muciniphila* has the potential to prevent obesity, diabetes, and atherosclerosis and increase the efficacy of cancer immunotherapy, this dysbiosis member raises the question “Could it be a repair function for HSIL and LSIL?”^(33,34).

Our findings regarding alpha diversity mirrored the previously reported pattern of changes in species richness and community composition of the vaginal microbiota along the progression of cervical lesions⁽³⁵⁾. In contrast, the absence of a statistically significant difference in beta diversity across the groups could be attributed to the application of the NMDS model in our analysis. To assess the robustness of this model, we also performed stress plot evaluations. We also included stress plot analyses in our research to understand the reliability of this model.

Study Limitations

Given the relatively small sample size, there is a possibility that the statistical power may have been insufficient. Therefore, the results should be approached and interpreted cautiously. Among the patients included in the study, only specific HPV types -HPV6, 16, 18, 31, 35, 42, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, and 68- were detected (Table S1), while other HPV types were not observed. This restricted HPV spectrum may limit the generalizability of our results and could hinder a comprehensive understanding of the relationship between vaginal microbiota composition and cervical intraepithelial

neoplasia. Future research with larger, more diverse cohorts, encompassing a broader range of HPV genotypes, is warranted to clarify whether the observed associations persist across different viral subtypes and to provide a more complete insight into this interaction.

Conclusion

The results of this study demonstrate that alterations in vaginal microbiota composition are associated with the severity of cervical lesions. However, further investigations are necessary to determine whether these microbial shifts contribute to the development of cervical lesions or arise as a consequence of the disease process.

Ethics

Ethics Committee Approval: The research protocol received ethical clearance from the Clinical and Interventional Research Ethics Committee of Yozgat Bozok University Research and Application Hospital, with official approvals granted on 25 August 2022 and 22 September 2022 (decision no: 2017-KAEK-189_2022.08.25_10, date: 28.08.2022 and decision no: 2017-KAEK-189_2022.09.22_06, date: 22.09.2022).

Informed Consent: All participants provided written informed consent.

Footnotes

Authorship Contributions

Surgical and Medical Practices: F.K., S.K., D.A.K., Concept: E.S.Y., Ç.A., M.B., Design: E.S.Y., Ç.A., M.B., A.E., Data Collection or Processing: Ç.A., M.B., E.Y.Ş., F.K., S.K., A.E., D.A.K., Analysis or Interpretation: M.B., F.K., S.K., D.A.K., T.O., Literature Search: Ç.A., M.B., E.Y.Ş., A.E., D.A.K., T.O., Writing: Ç.A., F.K., S.K., D.A.K., T.O.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This research was funded by Yozgat Bozok University Scientific Research Projects Unit (project ID numbers: 1066/1067; project codes: TÇD-2023-1066/TÇD-2023/1067).

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Table S1. Distribution of HPV genotypes by cervical pathology groups

Cervical pathology group	HPV 16/18 positive	Other high-risk HPV positive	Low-risk HPV positive	n
NILM	11	16	8	35
LSIL	11	9	8	28
HSIL	6	7	11	24
Total	87	32	27	87
NILM: No intraepithelial lesion or malignancy, LSIL: Low-grade squamous intraepithelial lesion, HSIL: High-grade squamous intraepithelial lesion, HPV: Human papillomavirus				

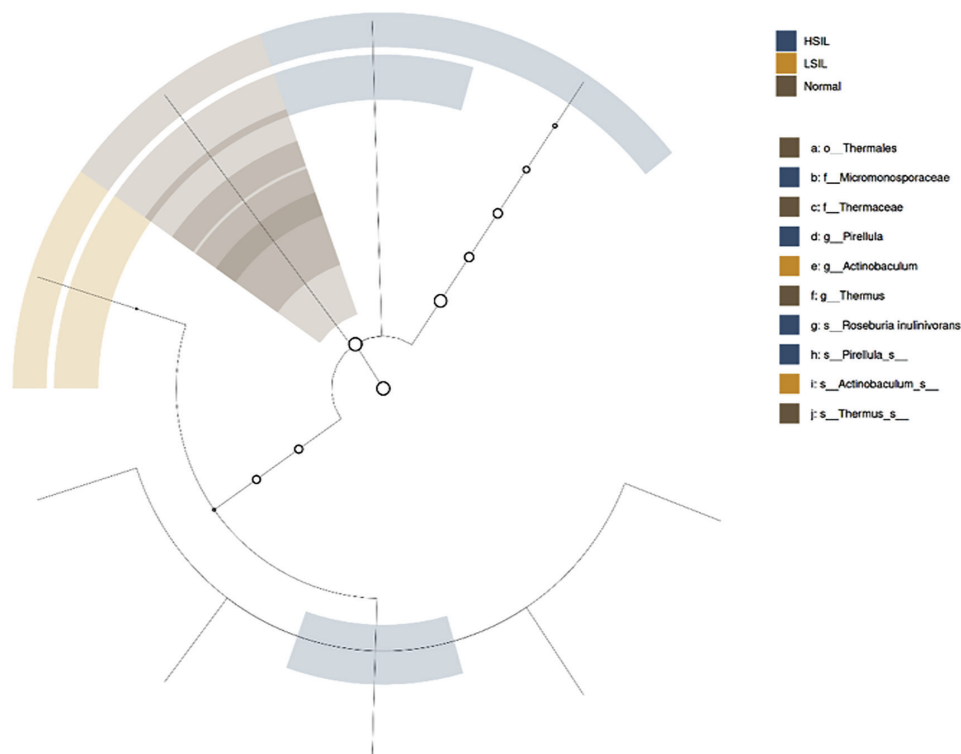


Figure S1. Linear discriminant analysis effect cladogram appearance according to groups

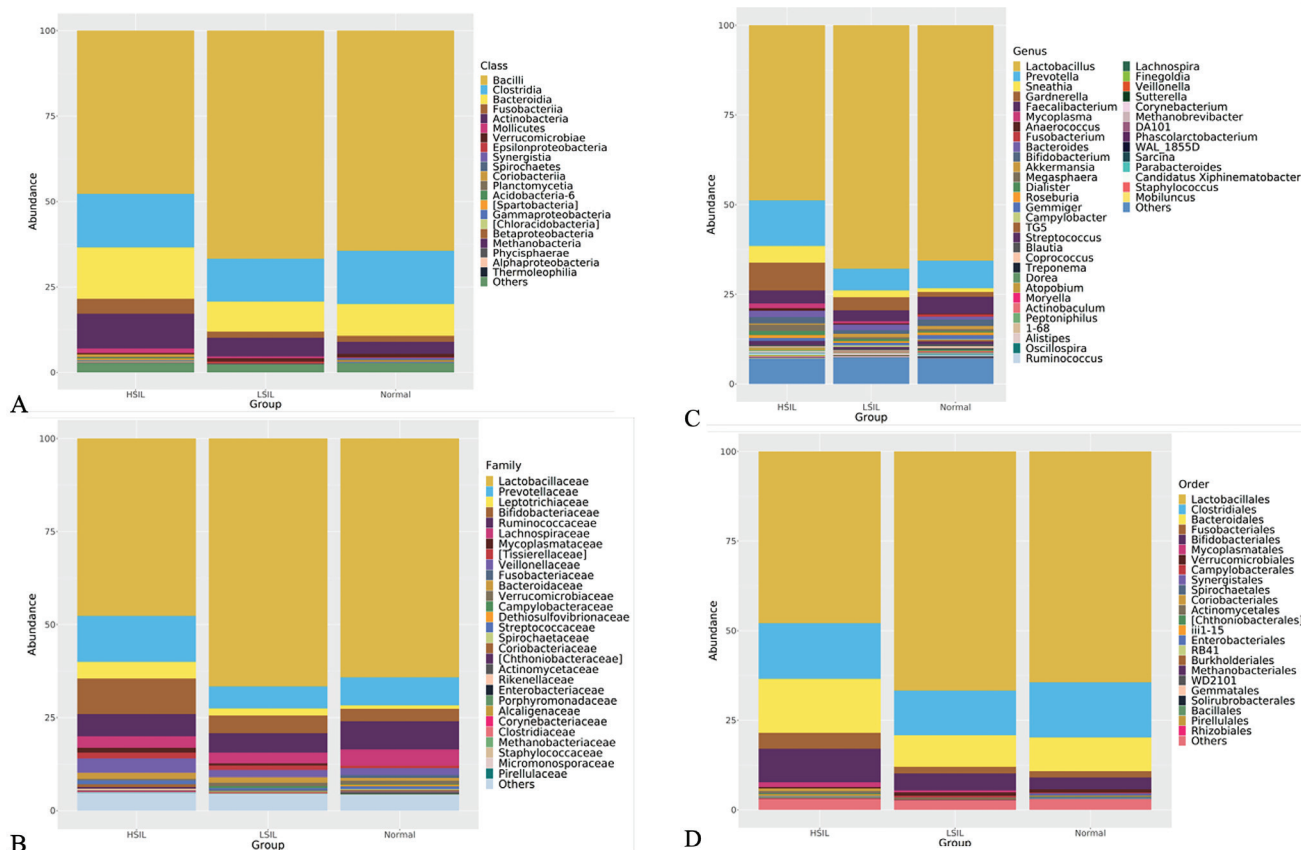


Figure S2A. Bacteria abundance distribution at class level; B: Bacteria abundance distribution at family level; C: Bacteria abundance distribution at genus level; D: Bacteria abundance distribution at order level