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Cell cycle gene expression and microbial profiles associated with HPV related cervical neoplasia and cervical microbiome shifts

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Background and objectives The improper regulation of the cell cycle is a significant risk factor for cervical cancer (CC), which is a serious health concern for women worldwide. The interaction between viral pathogens and microbiota with host cells could be vital in this dysregulation. The research aimed to explore changes in the transcription levels of *CDK2* and *CCNE1*, and how these changes relate to the HPV genotypes in women with cervical intraepithelial neoplasia (CIN) compared with non-infected healthy women. Additionally, microbiome and gene expression analyses were conducted in a subgroup of participants with HPV-16/18 infection in compare to non-infected healthy women. **Materials and methods** A cross-sectional study was conducted on women who attended cervical screenings at Yas Hospital in Tehran between March 2024 and May 2025. Three segments of exocervical biopsies were collected during examinations. Pathological analysis was performed to study histological changes, and gene expression analysis was conducted to detect relative transcriptional changes in *CDK2* and *CCNE1* levels using quantitative real-time polymerase chain reaction (qRT-PCR). Additionally, 16S rDNA sequencing was performed using the Illumina MiSeq platform to characterize differences in bacterial composition among the samples. Statistical analysis was conducted to investigate the correlation between infection status, cell cycle dysregulation, and microbial diversity. **Results** A total of 220 women (mean age 36.1 ± 8.33 years) were enrolled. Of these, 206 (93.6%) tested positive for HPV, while 15 (6.81%) served as HPV-negative individuals by PCR. Among the HPV-positive patients, HPV-16 and HPV-18 were detected in 30.5% and 11.8%, respectively; the remaining high-risk genotypes accounted for 50.9%, with HPV-66 being the most common (12.4%). *CCNE1* transcription was significantly higher in the infected group compared to the non-infected group (p-value = 0.001), in HPV-16-positive patients versus HPV-18-positive patients (p-value = 0.015), and in HPV-16-positive patients versus patients with other genotypes (p-value = 0.034). *CDK2* transcription was elevated in HPV-18-positive patients compared to non-infected individuals (p-value = 0.009), in HPV-16-positive patients compared to HPV-18-positive patients (p-value = 0.019), and in patients with different HPV types compared to HPV-18-positive patients (p-value = 0.001). No significant differences were observed in alpha (p-value = 0.89) or beta (p-value = 0.46) diversity. HPV-16 and HPV-18 infections led to notable changes in the cervical microbiome compared to the non-infected group, showing increased levels of *Prevotella_7*, *Megasphaera*, *Corynebacterium*, and *Limosilactobacillus*, while reducing *Lactobacillus*, *Gardnerella*, *Bacillus*, and *Streptococcus*. There were also genotype-specific patterns in *Bifidobacterium*, *Actinomyces*, and *Atopobium*. Although no significant correlations were found between the microbiome composition and *CCNE1*/*CDK2* transcription, descriptive analysis suggested a

potential correlation. Conclusion This study shows that HPV infection, especially high-risk genotypes, is linked to an increase in transcription of *CCNE1* and *CDK2*, indicating cell cycle dysregulation in cervical intraepithelial neoplasia. Although no significant difference in overall microbial diversity was detected between infected and non-infected individuals, a change in specific bacterial genera was observed that was correlated with alterations in the transcription of G1/S cell cycle mediators.

Keywords Cervical screening, HPV, Cyclin E1, CDK2, CIN, Biomarkers, Cervical microbiome

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Cervical cancer (CC) is the fourth most common cancer among women globally. This situation represents a serious public health issue, as it greatly endangers women's health. The incidence of this disease can be linked to various factors, such as early marriage, numerous pregnancies, repeated abortions, genetic predispositions, sexually transmitted infections, and—most importantly—persistent high-risk HPV infections[1].

A group of viruses known as HPVs impacts the cervix and other mucosal and skin regions. It has been determined that these are among the most common sexually transmitted infections (STIs) worldwide. There is a correlation between the presence of low-risk and high-risk strains of these infections and malignant tumors[2]. The risk of HPV infection for sexually active people is estimated to be more than 80% by the age of 45[3]. Despite the immune system's ability to eliminate the majority of infections, a significant proportion, ranging from 10% to 20%, may persist, thereby contributing to the development of cancerous growths[4]. The ongoing infection with certain HPV types, especially those linked to cancer, has been correlated with a higher likelihood of developing cancers in areas such as the throat, vulva, anus, vagina, cervix, and penis[5].

Cervical intraepithelial neoplasia (CIN) can be triggered by prolonged exposure to high-risk HPV strains, primarily due to the effects of the viral oncoproteins E6 and E7. The proteins in question have been shown to contribute to oncogenesis by altering DNA methylation and disrupting pathways associated with genomic stability, cell adhesion, immune evasion, and apoptosis. This, in turn, impairs the function of key tumor suppressors, including p53 and retinoblastoma (pRb)[6]. Although their precise functions in the viral life cycle remain to be elucidated, it is hypothesized that E6 and E7 play a pivotal role during the S phase of keratinocyte differentiation. This is believed to ensure the availability of host replication proteins, thereby facilitating the amplification of viral DNA[7]. The development of tumors in CC is significantly influenced by the HPV oncoproteins E6 and E7, primarily due to their inhibition of tumor suppressor activities. Specifically, E7 from both high- and low-risk HPV strains has been demonstrated to enhance *cyclin-dependent kinase 2 (CDK2)* activity and cyclin E expression, thereby facilitating progression of the cell cycle through the S and G2/M stages[8].

The processes of gene expression, cell division, growth, and programmed cell death are all contingent upon serine/threonine protein kinases, known as *CDKs*. *CDK2* is one of the subtypes that has been the subject of the most extensive research[9]. The CDK2 complex, which consists of *cyclins E* (*CCNE1*) and A (*CCNA*), is a regulatory element in the process of cellular proliferation, specifically governing the transition from the G1 phase to the S phase in mammalian cells[10]. HPV has likely developed mechanisms that can disrupt *CDK2* activity[11].

The mechanisms that govern the clearance of HPV or its persistence within the body remain to be fully elucidated. The elements associated with the persistent presence of HPV and its progression to cervical dysplasia or cancer include the structure of the epithelial surface, immune reactions in the mucosa, co-infections, as well as the cervical microbiome and its microenvironment[12]. The cervical microbiome (CVM), which is frequently dominated by *Lactobacillus* species, plays a pivotal role in maintaining vaginal health. A decrease in the amount of *Lactobacillus* species has been shown to result in dysbiosis of the cardiovascular microbiome. This dysbiosis has been observed to promote tumor growth by producing genotoxins, disrupt the immune system, and induce chronic inflammation[13,14]. In women diagnosed with CIN, elevated levels of *Gardnerella* have been observed, accompanied by a significant decrease in protective *Lactobacillus* species. The presence of a microbial imbalance has been demonstrated to contribute to the progression of CIN. Furthermore, an increased diversity of microbes has been observed in patients, indicating a multifaceted bacterial interaction in this condition[15]. A paucity of studies has been observed in the investigation of the relationship between cervical microbiome and CIN. Despite the plethora of studies that have established a correlation between the composition of the cervical microbiota and the persistence of HPV or cervical neoplasia, the precise microbial species implicated and the distinguishing characteristics between patients with CC and healthy individuals remain to be fully elucidated[1].

The present study aims to explore the levels of *CDK2* and *CCNE1* gene expression in CIN associated with high-risk HPV infections and to examine the potential impact of the cervicovaginal microbiota on the persistence of the infection and the progression of the disease. This study offers a more comprehensive understanding of the mechanisms underlying cervical carcinogenesis.

Results

Characteristics and profiles of HPV-positive patients

In this research, data were first gathered from 418 female participants. Several participants were excluded from the study due to recent antibiotic use or a diagnosis of bacterial vaginosis, resulting in 220 participants for the analysis. None of the participants received any HPV vaccination, as indicated by the questionnaire. Among all participants, 206 had HPV infection; the remaining 15 constituted the PCR-confirmed HPV-negative group. Participants' ages ranged from 20 to 57 years, with a mean age of 36.1 years (SD = 8.32). Of the HPV-positive subjects, 113 (51.4%) had other high-risk HPV genotypes, 26 (11.8%) had HPV genotype 18, and 67 (30.5%) had HPV genotype 16. The most frequently detected genotypes were HPV-16, 52, 31, and 66, whereas HPV-58, 51, and 61 were less common. Among the HPV-positive participants, 45.9% reported smoking and 28.9% reported alcohol consumption. Among HPV-positive individuals, 84 had CIN-I, 54 had CIN-II/III, and 82 had normal biopsies. Smoking rates were 30.5% for normal biopsies, 48.8% for CIN-I, and 64.8% for CIN-II/III. Alcohol use was 23.2% for normal biopsies, 28.6% for CIN-I, and 38.9% for CIN-II/III. Statistical analysis showed that CIN-I individuals were significantly more likely to smoke and drink than those with normal biopsies (p-*value* = 0.001). A summary of the demographic characteristics of the study population is presented in Table 1.

Expression of *CDK2* and *CCNE1* is associated with increased CIN grade

The gene expression analysis showed distinct group patterns. The mean fold change expression levels of the *CCNE1* gene were 1186.6, 1714.0, 2766.6, and 242.0 in the HPV-16, 18, other high-risk genotypes, and non-infected groups, respectively. Similarly, the mean fold change expression levels of the *CDK2* gene were 17.9, 25.8, 11.0, and 8.0 in these groups, respectively. Box plots (Fig. 1) illustrate these findings.

When compared to the non-infected group, the expression of the *CCNE1* gene was significantly higher in the HPV-16, HPV-18, and other high-risk genotype groups, as determined by statistical analysis using the Kruskal-Wallis test (p-value < 0.05). Additionally, the HPV-18 group exhibited significantly higher *CDK2* expression than the non-infected group. Furthermore, the HPV-18 group showed higher expression of *CDK2* than other high-risk genotypes, and the HPV-16 group showed higher expression than the HPV-18 group in both genes (p-value < 0.05). Although a noticeable upward trend in *CDK2* expression was observed in the other groups, these differences did not reach statistical significance. These findings suggest that alterations in *CCNE1* and *CDK2* expression may be associated with HPV oncogenic activity and could serve as molecular markers for distinguishing HPV-related lesions or guiding targeted therapeutic strategies in the future (Table 2). ROC curves for CCNE1 and CDK2 expression levels were generated to distinguish HPV-16, HPV-18, and other HPV genotypes from the non-infected group. These analyses showed the corresponding AUC and p-values, indicating

	HPV-16 *n* (%)	HPV-18 *n* (%)	Other HR-HPV *n* (%)	Non-infected *n* (%)	Total *N* (%)
Total	67 (30.5%)	26 (11.8%)	113 (51.4%)	15 (6.8%)	221
Biopsy					
Normal	19 (28.4%)	4 (15.4%)	44 (38.9%)	15 (100%)	82 (37.3%)
CIN I	32 (47.8%)	11 (42.3%)	41 (36.3%)	0	84 (38.2%)
CIN II/III	16 (23.8%)	11 (42.3%)	28 (24.8%)	0	54 (24.5%)
Pap smear					
NILM	41 (61.2%)	10 (38.5%)	69 (61.1%)	13 (86.7%)	132 (60.0%)
ASCUS	15 (22.4%)	11 (42.3%)	26 (23.0%)	0	52 (23.6%)
LSIL	10 (14.9%)	4 (15.4%)	13 (11.5%)	1 (6.7%)	28 (12.7%)
HSIL	1 (1.5%)	1 (3.9%)	5 (4.4%)	1 (6.7%)	8 (3.6%)
Smoking					
Non-smoker	31 (46.3%)	12 (46.2%)	61 (54.0%)	15 (100%)	119 (54.1%)
Smoker	36 (53.7%)	14 (53.9%)	52 (46.0%)	0	101 (45.9%)
Alcohol					
Non-drinker	43 (64.2%)	13 (50.0%)	85 (75.2%)	15 (100%)	156 (70.9%)
Drinker	24 (35.8%)	13 (50.0%)	28 (24.8%)	0	64 (29.1%)
Mean ± SD					
Age (years)	34.9 ± 8.10	36.1 ± 8.34	36.7 ± 8.51	36.8 ± 8.04	36.1 ± 8.33
Duration (months)	16.5 ± 19.6	11.9 ± 9.1	21.3 ± 28.3	–	–
Median					
Age (years)	34	37	36	35	35
Duration (months)	12	9.5	12	–	12

Table 1. Descriptive information for infected and healthy non-infected participants in this study. NILM: Negative for Intraepithelial Lesion or Malignancy; ASCUS: Atypical Squamous Cells of Undetermined Significance; LSIL: Low-grade Squamous Intraepithelial Lesion; HSIL: High-grade Squamous Intraepithelial Lesion; CIN I: Cervical Intraepithelial Neoplasia grade I; CIN II: Cervical Intraepithelial Neoplasia grade II; CIN III: Cervical Intraepithelial Neoplasia grade III

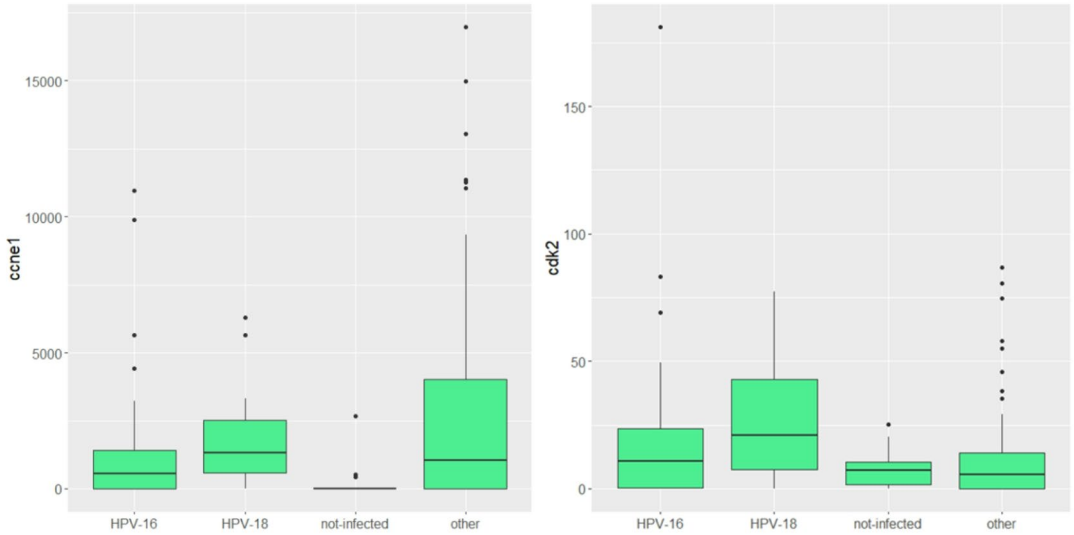

Fig. 1. Boxplots of differential transcription of *CDK2* and *CCNE1* in cervical biopsies of HPV-infected and non-infected women..

Group 1	Group 2	*CCNE1*		*CDK2*	
		Fold change	*p*-value	Fold change	P-value
HPV-16	not-infected	787.5	**0.001**	585	0.325
HPV-18	not-infected	349.5	**0.001**	292.5	**0.009**
Other high-risk Genotypes[a.]	not-infected	329	**0.001**	883.5	0.748
HPV-16	HPV-18	585	**0.015**	595.5	**0.019**
HPV-16	Other high-risk Genotypes	0.304	**0.034**	4344	0.078
HPV-18	Other high-risk Genotypes	1503	0.800	2153.5	**0.001**

Table 2. Comparison of CCNE1 and CDK2 gene expression levels across Normal, CIN I, and CIN II/III biopsy groups. *The significance level of 0.05 is considered. [a].Other high-risk genotypes were included 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68

the potential of these genes as diagnostic markers for differentiating high-risk HPV types from the non-infected group (Fig. 2).

Expression of *CDK2* and *CCNE1* is associated with increased CIN grade

The relationship between gene expression and various biopsy categories was assessed. Statistical evaluations indicated that *CCNE1* expression was notably lower in the non-infected group compared to the CIN-I and CIN-II/III groups (p-value < 0.001). A significant increase in *CCNE1* expression was observed when comparing the CIN-I and CIN-II/III groups (p-value = 0.017). Likewise, *CDK2* expression was significantly diminished in the non-infected group compared to the CIN-I and CIN-II/III groups (p-value < 0.001 and p-value = 0.006, respectively). While an increase in *CDK2* expression was observed in the CIN-II/III group compared to the CIN-I group, this difference did not reach statistical significance (p-value = 0.062). These results suggest that the upregulation of *CCNE1* and *CDK2* expression is associated with the progression of CIN and may play a crucial role in regulating the cell cycle and facilitating abnormal cell proliferation during the precancerous changes in cervical tissue (Table 3).

Association between cervical microbiome composition and high-risk HPV-16/18 infections

Distribution of OTUs (operational taxonomic units) across non-infected, HPV-16, and HPV-18 groups

In this study, operational taxonomic units (OTUs), defined as clusters with greater than 97% similarity in 16S rDNA sequences, were analyzed at the phylum level. Overall sequencing reads indicated that the non-infected group (H0) exhibited the highest average OTU abundance, while HPV-16 and HPV-18 positive groups (H16 and H18) showed lower abundances. These observations reflect changes in microbial community composition rather than overall diversity. In H0, the cervical microbiota was primarily composed of *Firmicutes* (39.0%), *Deinococcota* (23.4%), *Proteobacteria* (17.9%), and *Actinobacteriota* (14.7%), with minor representation from *Bacteroidota* (1.77%), *Chloroflexi* (1.35%), *Aenigmarchaeota* (0.55%), *Acidobacteriota* (0.35%), and *Crenarchaeota* (0.31%). In

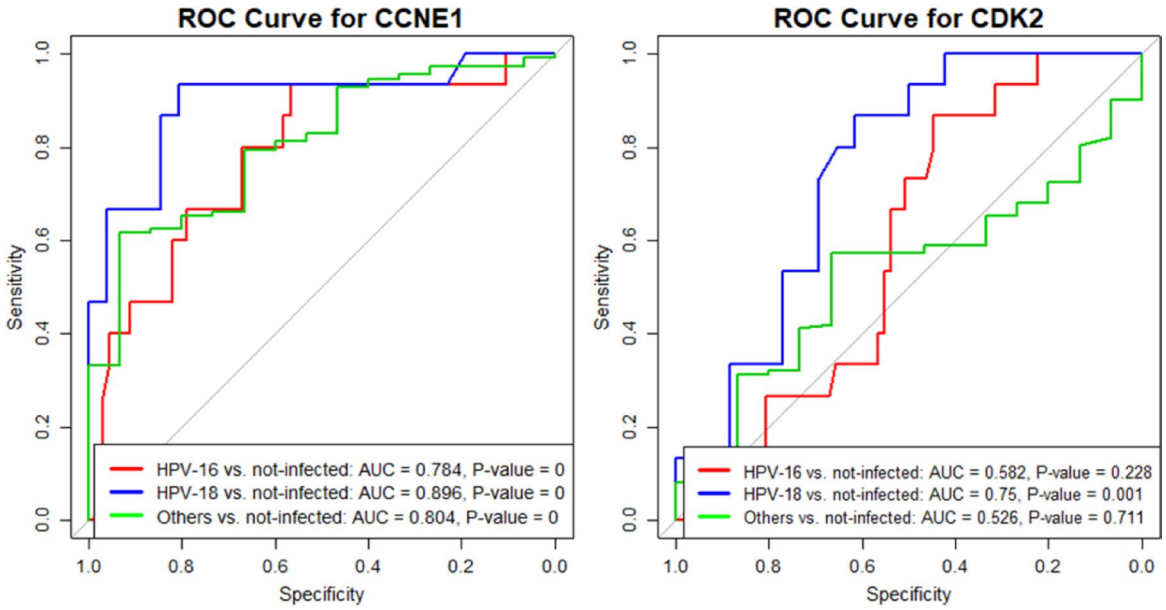

Fig. 2. ROC curves for *CCNE1* and *CDK2* expression levels in distinguishing HPV-16, HPV-18, and other HPV types from non-infected samples, showing corresponding AUC and p-values.

Biopsy group comparison	*CCNE1*		*CDK2*	
	Test statistic	*p*-value [a.]	Test statistic	*p*-value
Normal vs. CIN I	1388.5	**0.001**	1759.5	* **0.001**
Normal vs. CIN II/III	644.5	**0.001**	831	* **0.001**
CIN I vs. CII/III	1721.5	**0.017**	1839.5	0.062

Table 3. Comparison of *CCNE1* and *CDK2* Gene Expression Levels Across Normal, CIN I, and CIN II/III Biopsy Groups [a.]The significance level of 0.05 is considered

H16, *Proteobacteria* (34.3%) and *Firmicutes* (31.5%) were the most abundant, followed by *Deinococcota* (17.9%), *Bacteroidota* (7.62%), and *Actinobacteriota* (7.02%), while less abundant phyla included *Chloroflexi* (0.72%), *Gemmatimonadota* (0.20%), *Spirochaetota* (0.18%), *Aenigmarchaeota* (0.17%), and *Cyanobacteria* (0.12%). In H18, *Firmicutes* dominated (48.6%), followed by *Proteobacteria* (31.8%), *Deinococcota* (10.7%), *Actinobacteriota* (4.72%), and *Bacteroidota* (3.52%), with minor phyla including *Chloroflexi* (0.44%), *Gemmatimonadota* (0.06%), *Myxococcota* (0.06%), *Cyanobacteria* (0.04%), and *Aenigmarchaeota* (0.03%). Comparative analysis indicated that *Firmicutes* abundance was higher in H18 compared to H0 and H16, whereas Proteobacteria reached its highest proportion in H16. *Actinobacteria* and *Deinococci* exhibited a reduced relative abundance in HPV-positive groups, particularly in H18, whereas *Bacteroidota* showed an increased relative abundance compared to the non-infected group. Overall, these findings highlight that HPV infection, especially HPV-18, is associated with observable shifts in the cervical microbial community composition rather than overall diversity (Fig. 3).

Alpha diversity comparison across study groups

Analysis of alpha diversity using Shannon indices (Fig. 4-A) revealed no significant variation among the non-infected, HPV-16, and HPV-18 groups, as determined by the Kruskal–Wallis test ($H = 0.4230$, *p-value* $= 0.8094$). Similarly, pairwise comparisons showed no statistically significant differences between the HPV-16 and non-infected *($H = 0.1020$, p-value $= 0.7494$)*, HPV-18 and the non-infected *($H = 0.0367$, p-value $= 0.4822$)*, and HPV-18 and HPV-16 *($H = 0.036$, p-value* $= 0.8480$). Taken together, these results indicate that microbial richness and diversity were largely comparable across groups, and this pattern remained consistent even when analyzed at the genus level of taxonomic classification.

Beta diversity analysis

PERMANOVA analysis was conducted to assess differences in beta diversity among the non-infected, HPV-16, and HPV-18 groups. The results showed no statistically significant variation in overall community composition (pseudo-F = 0.9777, *p*-value = 0.4630). The analysis included 21 samples distributed across three groups, and the test statistic (pseudo-F) did not indicate measurable compositional divergence between the groups when beta diversity was estimated at the genus level. This result was also confirmed using principal coordinate analysis (PCoA) of unweighted UniFrac distances (Figs. 4B and C).

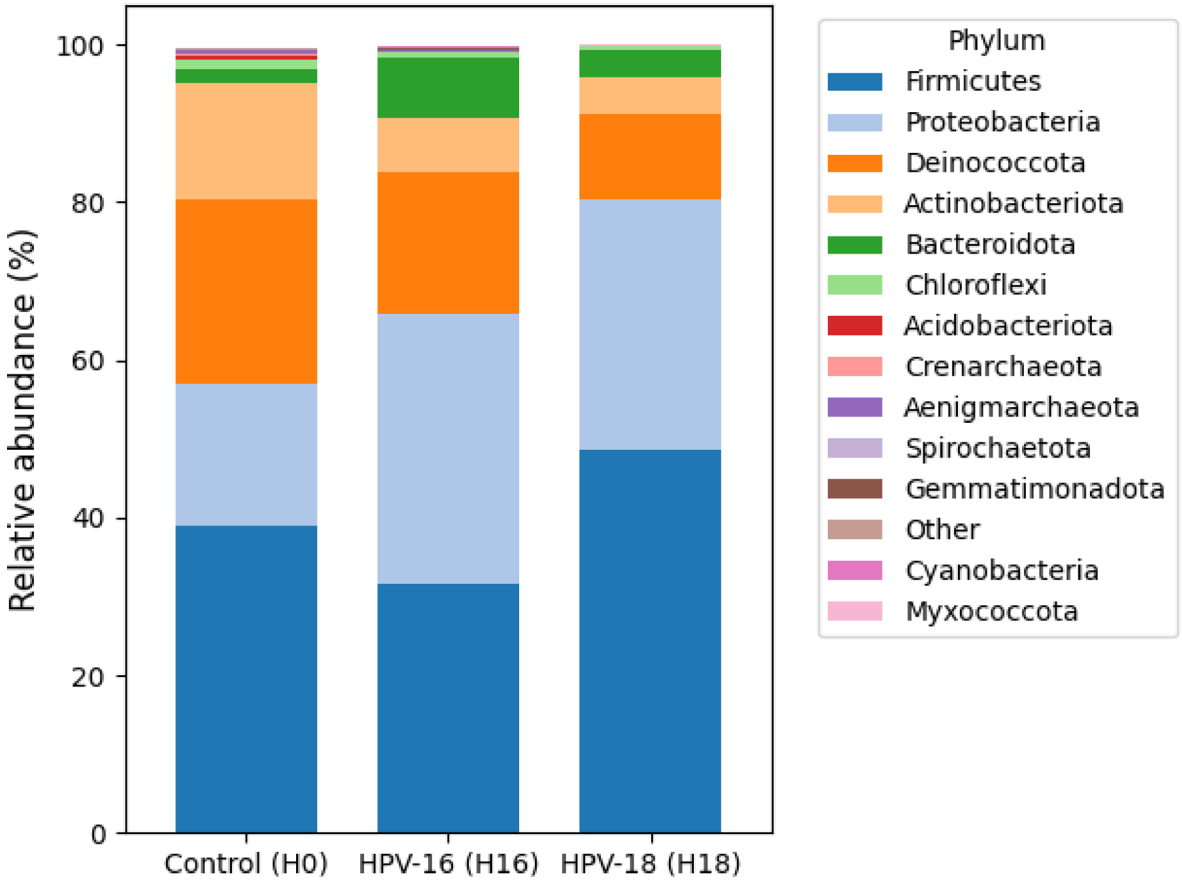

Fig. 3. The stacked bar chart displays the relative abundance of bacterial phyla in cervical samples from non-infected (H0), HPV-16 (H16), and HPV-18 (H18) groups. *Firmicutes*, *Proteobacteria*, and *Deinococcota* were the most prevalent phyla, with varying proportions among the groups.

Comparison of bacterial abundance at genus and family levels

Analysis of the cervical microbiome across the non-infected, HPV-18, and HPV-16 groups revealed distinct genus- and family-level patterns, although Kruskal–Wallis tests showed no statistically significant differences ($p > 0.05$) (Fig. 5; Table 4). Additionally, the LDA Score chart was analyzed and is presented in Fig. 6.

At the genus level, *Lactobacillus* was dominant in all groups, accounting for 34.52% ($N = 61,394$) in non-infected individuals, 66.92% ($N = 173,253$) in HPV-18, and 23.19% ($N = 49,335$) in HPV-16, representing a 1.9-fold enrichment in HPV-18 and a 0.67-fold decrease in HPV-16 relative to non-infected individuals. *Gardnerella* sharply decreased in HPV-16 (0.06%, $N = 124$) and HPV-18 (2.77%, $N = 7,160$) compared to non-infected individuals (25.45%, $N = 45,256$), corresponding to 0.002- and 0.11-fold changes, respectively.

Prevotella_7 was explicitly enriched in HPV-16 (11.03%, $N = 23,472$) and showed a marked increase in HPV-18 (higher than in HPV-16), whereas it was absent in the HPV-negative samples. *Stenotrophomonas* showed a substantial increase in HPV-18 and minimal change in HPV-16. Other increased genera included *Megasphaera*, *Corynebacterium*, *Nocardiodes*, *Prevotella*, and *Limosilactobacillus*, which were elevated in both HPV-16- and HPV-18-infected individuals compared to those who were HPV-negative. Additionally, Acinetobacter, *Chryseobacterium*, *Delftia*, and *Pseudomonas* showed minor increases, with the increase more pronounced in HPV-18.

Conversely, decreased genera included *Lactobacillus* (both genotypes, more pronounced in HPV-18), *Gardnerella* (sharply decreased in HPV-18 and moderately in HPV-16), *Prevotella_9*, *Gemella*, *Thermaerobacter*, *Atopobium*, *Microbacterium*, *Actinomyces*, *Bacillus*, and *Streptococcus* (both HPV groups). Some genera exhibited contrasting patterns between HPV-16 and HPV-18, including *Bifidobacterium* (decreased in HPV-16 but increased in HPV-18), *Actinomyces* (decreased in HPV-16 but remained almost unchanged in HPV-18), and *Atopobium* (decreased only in HPV-18).

At the family level, *Lactobacillaceae* was enriched in HPV-18 (33.82%, $N = 174,613$) compared to non-infected individuals (14.13%, $N = 62,312$) and HPV-16 (11.37%, $N = 53,393$), corresponding to a 2.4-fold increase in HPV-18 and a 0.8-fold decrease in HPV-16. *Bacillaceae* (18.42%, $N = 86,450$) and *Thermaceae* (19.13%, $N = 89,775$) were more prevalent in HPV-16 compared to non-infected individuals and HPV-18. Other families, including *Sphingomonadaceae*, *Bifidobacteriaceae*, *Corynebacteriaceae*, *Xanthobacteraceae*, and *Comamonadaceae*, showed moderate enrichment in HPV-infected groups. In contrast, low-abundance families such *as Muribaculaceae*, *Dermabacteraceae*, and *Clostridiaceae* were nearly absent in HPV-positive samples.

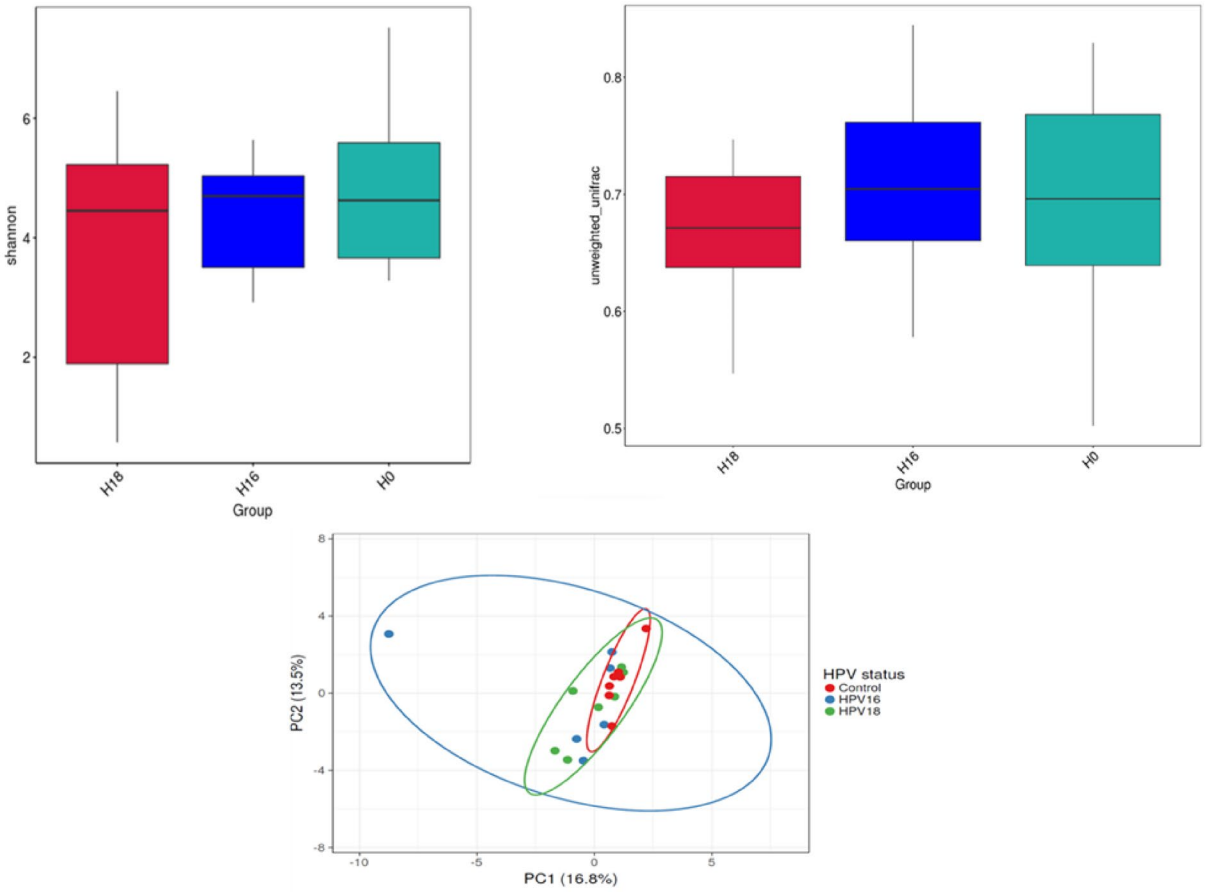

Fig. 4. **(A)** HPV-16 (H16) and HPV-18 (H18) groups exhibit lower microbial alpha diversity (Shannon Entropy) than the non-infected group (H0). **(B)** Beta diversity analysis shows higher unweighted UniFrac distances in H16 and H0 groups, indicating greater microbial dissimilarity than H18. **(C)** Beta-diversity analysis showed that patients with HPV-18 exhibited greater differences compared to HPV-16, in terms of the taxa identified in the non-infected group.

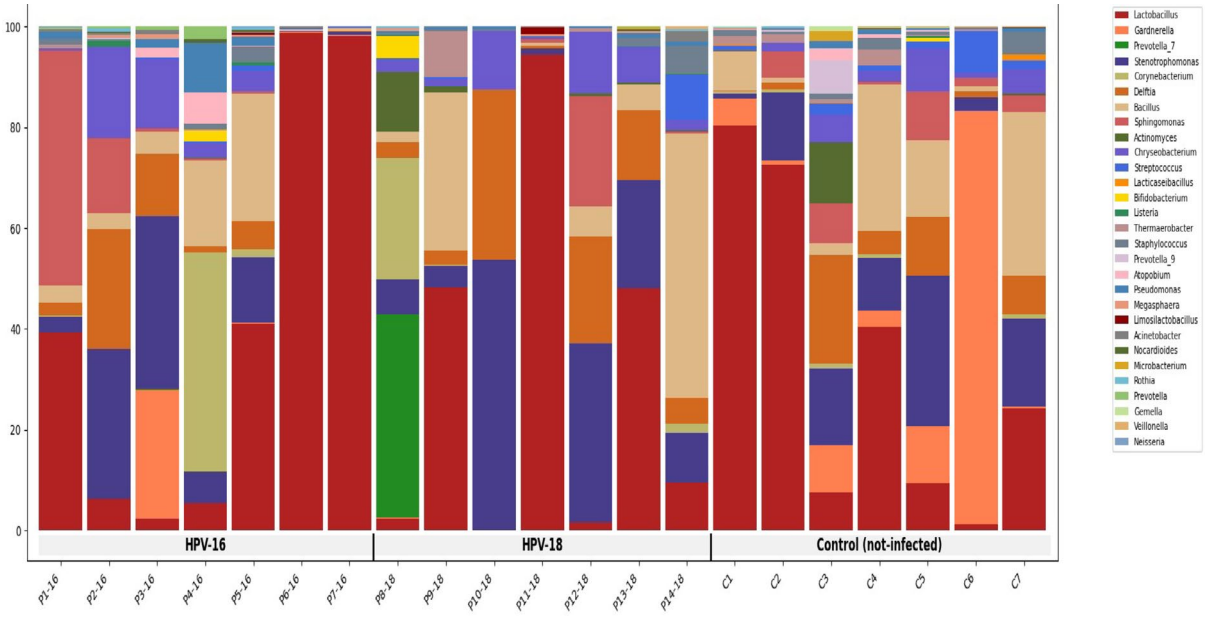

Fig. 5. The relative abundance of various bacterial genera in HPV-16, HPV-18, and non-infected groups.

Taxonomy	HPV-16		HPV-18		Not-infection			
Genus	*N*	%	*N*	%	*N*	%	Squared	*p*-value
Lactobacillus	49,335	23.19%	173,253	66.92%	61,394	34.52%	20	0.4579
Gardnerella	124	0.06%	7160	2.77%	45,256	25.45%	17.321	0.185
Prevotella_7	23,472	11.03%	90	0.03%	0	0.00%	5.0893	0.4051
Stenotrophomonas	46,030	21.64%	22,671	8.76%	21,124	11.88%	20	0.4579
Corynebacterium	14,391	6.77%	2410	0.93%	620	0.35%	16.837	0.2069
Delftia	27,787	13.06%	12,319	4.76%	8631	4.85%	20	0.3946
Bacillus	15,079	7.09%	11,162	4.31%	14,083	7.92%	20	0.4579
Sphingomonas	6424	3.02%	11,854	4.58%	7303	4.11%	16.19	0.5104
Actinomyces	7190	3.38%	42	0.02%	1775	1.00%	8.5714	0.8045
Chryseobacterium	12,515	5.88%	10,284	3.97%	5003	2.81%	20	0.4579
Streptococcus	1886	0.89%	567	0.22%	5230	2.94%	17.143	0.4447
Lacticaseibacillus	0	0.00%	0	0.00%	198	0.11%	5	0.1718
Bifidobacterium	2545	1.20%	95	0.04%	215	0.12%	5.7143	0.4559
Listeria	108	0.05%	492	0.19%	63	0.04%	4.375	0.4968
Thermaerobacter	602	0.28%	125	0.05%	1840	1.03%	14.286	0.2828
Staphylococcus	2073	0.97%	1265	0.49%	1695	0.95%	19.286	0.4386
Prevotella_9	9	0.00%	72	0.03%	1169	0.66%	6.0714	0.2993
Atopobium	0	0.00%	832	0.32%	484	0.27%	11.868	0.105
Pseudomonas	968	0.46%	1706	0.66%	564	0.32%	20	0.3946
Megasphaera	684	0.32%	560	0.22%	18	0.01%	9.0476	0.4329
Limosilactobacillus	362	0.17%	112	0.04%	32	0.02%	4.3697	0.3583
Acinetobacter	439	0.21%	686	0.26%	228	0.13%	19.048	0.2662
Nocardioides	168	0.08%	144	0.06%	15	0.01%	8.9796	0.2541
Microbacterium	82	0.04%	35	0.01%	271	0.15%	4.3697	0.3583
Rothia	218	0.10%	364	0.14%	255	0.14%	5	0.4159
Prevotella	137	0.06%	532	0.21%	34	0.02%	14.464	0.272
Gemella	0	0.00%	0	0.00%	193	0.11%	9.375	0.09501
Veillonella	79	0.04%	33	0.01%	145	0.08%	8	0.2381
Neisseria	0	0.00%	36	0.01%	6	0.00%	4.3651	0.2246
Family								
Lactobacillaceae	53,393	11.37%	174,613	33.82%	62,312	14.13%	20	0.4579
Sphingomonadaceae	6460	1.38%	62,870	12.18%	7702	1.75%	19.048	0.3889
Bifidobacteriaceae	2669	0.57%	7671	1.49%	45,471	10.31%	18.857	0.2761
Thermaceae	89,775	19.13%	58,435	11.32%	116,838	26.50%	20	0.4579
Bacillaceae	86,450	18.42%	62,594	12.13%	103,376	23.45%	20	0.4579
Enterobacteriaceae	51,166	10.90%	21,916	4.25%	16,169	3.67%	20	0.4579
Xanthobacteraceae	19,049	4.06%	32,338	6.26%	10,359	2.35%	20	0.3946
Prevotellaceae	24,299	5.18%	668	0.13%	1276	0.29%	14	0.4497
Xanthomonadaceae	51,338	10.94%	26,770	5.19%	26,693	6.05%	20	0.3946
Planococcaceae	0	0.00%	14,582	2.82%	365	0.08%	5.7143	0.2215
Corynebacteriaceae	14,391	3.07%	2474	0.48%	734	0.17%	16.837	0.2069
Comamonadaceae	31,882	6.79%	15,256	2.96%	13,486	3.06%	20	0.4579
Actinomycetaceae	7190	1.53%	227	0.04%	2252	0.51%	11.429	0.6521
Weeksellaceae	12,515	2.67%	10,747	2.08%	5003	1.13%	20	0.4579
Microbacteriaceae	0	0.00%	684	0.13%	4208	0.95%	12.976	0.1637
Streptococcaceae	63	0.01%	453	0.09%	5238	1.19%	14.026	0.1718
Staphylococcaceae	1874	0.40%	4544	0.88%	309	0.07%	11.169	0.3445
Propionibacteriaceae	2229	0.47%	2028	0.39%	4694	1.06%	20	0.4579
Listeriaceae	108	0.02%	364	0.07%	2247	0.51%	4.375	0.4968
Micrococcaceae	1515	0.32%	4540	0.88%	960	0.22%	19.048	0.3889
Lachnospiraceae	1250	0.27%	92	0.02%	2329	0.53%	11.169	0.3445
Thermomicrobiaceae	3189	0.68%	2263	0.44%	2553	0.58%	15.952	0.3852
Moraxellaceae	693	0.15%	1901	0.37%	422	0.10%	19.048	0.3258
Paenibacillaceae	3715	0.79%	2771	0.54%	4195	0.95%	18.929	0.2724
Burkholderiaceae	1658	0.35%	1528	0.30%	720	0.16%	14.286	0.5774
Continued								

Taxonomy	HPV-16		HPV-18		Not-infection			
Genus	*N*	%	*N*	%	*N*	%	Squared	*p*-value
Muribaculaceae	0	0.00%	273	0.05%	31	0.01%	6.0952	0.2971
Dermabacteraceae	0	0.00%	1305	0.25%	0	0.00%	5	0.1718
Pseudomonadaceae	968	0.21%	1021	0.20%	564	0.13%	20	0.2742
Veillonellaceae	895	0.19%	374	0.07%	251	0.06%	8.5714	0.4777
Peptostreptococcaceae	671	0.14%	425	0.08%	157	0.04%	7.2619	0.6099
Clostridiaceae	0	0.00%	508	0.10%	0	0.00%	5	0.1718

Table 4. Statistical comparison of cervical microbiota using Kruskal–Wallis Test.

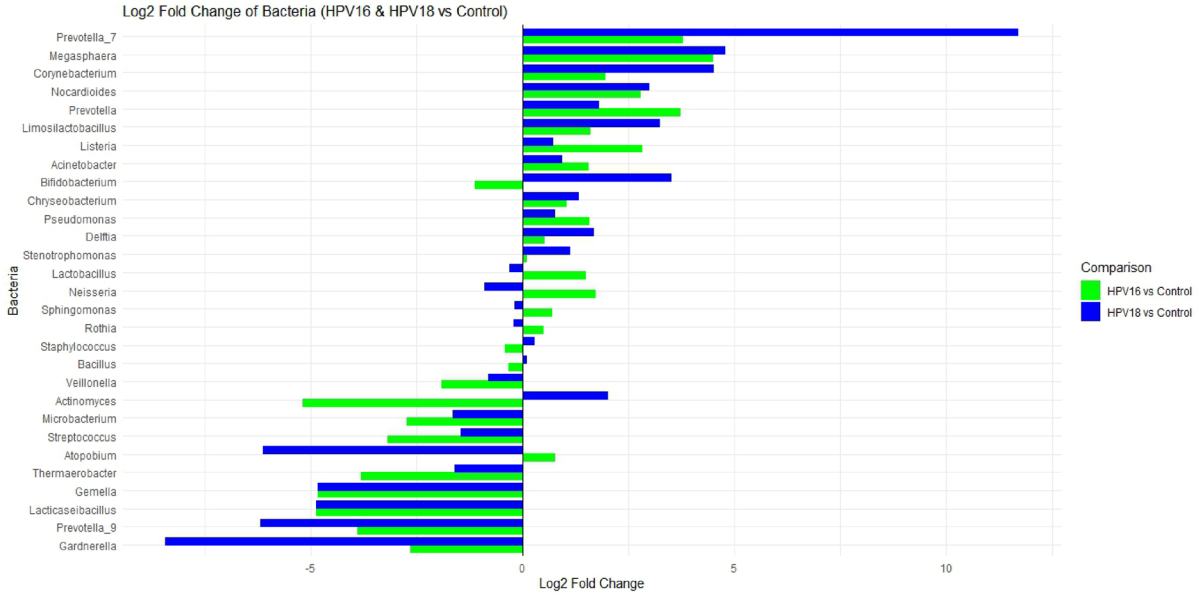

Fig. 6. Linear discriminant analysis (LDA) scores of bacterial genera across study groups. LDA was performed to identify taxa that most strongly distinguish the cervical microbiome.

Gene–microbiome correlation patterns across Non-infected, HPV-18, and HP-V16 groups

In the analysis of correlations between *CCNE1* and *CDK2* expression and the cervical microbiome of HPV-positive individuals, None of the relationships achieved statistical significance (all *p-values* > 0.05). Therefore, the reported associations should be interpreted as descriptive trends rather than definitive evidence. Despite the lack of statistical significance, the descriptive analysis and the heatmap presented in Fig. 7 revealed notable group-specific patterns. In HPV-18, *CCNE1* showed a positive correlation with *Bacillus* ($r = 0.93$) and a negative correlation with *Lactobacillus* ($r = -0.18$); in contrast, *CDK2* predominantly displayed a negative correlation with *Sphingomonas* ($r = -0.68$). In HPV-16, *CCNE1* correlated positively with *Streptococcus* ($r = 0.93$) and *CDK2* with *Bifidobacterium* ($r = 0.95$). Genera such as *Gemella* and *Veillonella* also showed positive correlations with both genes.

Population differences were observed between HPV-16 and HPV-18 for *Bifidobacterium*, *Lactobacillus*, and *Actinomyces*. Descriptively, in HPV-16, *Bifidobacterium* positively correlated with *CDK2* ($r = 0.95$), whereas in HPV-18, *Lactobacillus* negatively correlated with *CCNE1* ($r = -0.18$) and *Actinomyces* positively correlated with *CCNE1* ($r = 0.99$). Although these associations were not statistically significant, these trends suggest that genotype-specific microbiome composition may influence cell cycle gene expression patterns.

Discussion

This research highlights the complex interactions between host gene expression, the composition of the cervicovaginal microbiome, and high-risk HPV genotypes, all of which collectively contribute to the onset of cervical neoplasia. Comprehension of these interactions is imperative to elucidating the microbiological and molecular mechanisms underlying the development of CC. This deeper comprehension will ultimately inform the development of more effective diagnostic and preventive measures.

In this study, cervical tissue samples from women showed various HPV genotypes. Of 206 women who tested positive for HPV, 30.5% had HPV-16, 11.8% had HPV-18, and 50.9% had other high-risk types. The most common types were HPV-16, 66, 52, and 31, while HPV-61, 58, and 51 were less frequent. In the global

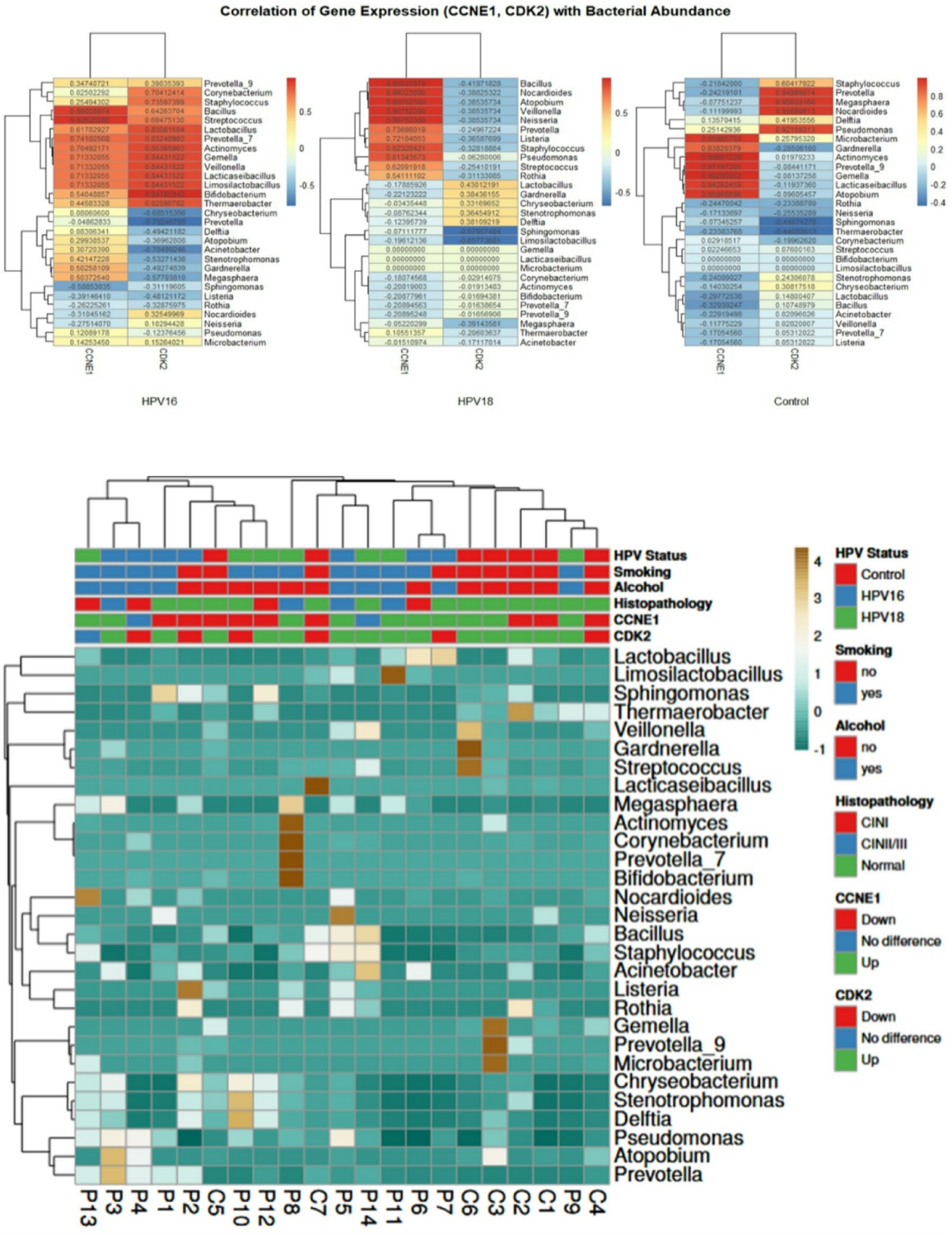

Fig. 7. Heatmaps of the correlation between the cervical microbiome at the genus level and cell-cycle differentially expressed genes, as well as histopathological and demographic characteristics. (**A**): Heatmaps showing correlations between gene expression (*CCNE1*, *CDK2*) and bacterial abundance in HPV-16, HPV-18, and non-infected groups. The dendrograms were generated using R with the heatmap library. (**B**): relative abundance of bacterial species in samples from three groups: P1-P7 (HPV-16), P7-P14 (HPV-18), P15-P21 (non-infected). The dendrograms were generated using R with the heatmap library.

context, the most prevalent HR-HPV genotypes include HPV-16, 18, 45, 52, 31, and 58. It has been established that approximately 70% of cases of CC are attributable to HPV-16 and 18. HPV 58, 52, 31, and 45 have also been identified as prevalent in East Asia, certain regions of Europe, Latin America, and Africa[16,17]. The increase in HPV infections in Iran and other countries, including those in sub-Saharan Africa, is a subject of concern[18] and is attributed to early sexual partnerships, changing societal norms, lack of awareness, and misuse of preventive measures. The factors mentioned above underscore a matter of grave public health concern that demands the immediate attention and education of the public[19]. These results diverge marginally from those observed in other countries, a discrepancy that can be attributed to variations in sample types, geographic regions, and methods for detecting HPV. Furthermore, the high cost of HPV testing in Iran has been identified as a significant barrier to access for a large number of women.

In HPV-positive subjects, there were 82 normal biopsies, 84 CIN-I cases, and 55 CIN-II/III cases. Smoking rates were 30.5% for normal biopsies, 48.8% for CIN-I, and 64.8% for CIN-II/III. Alcohol rates were 23.2%, 28.6%, and 38.9%, respectively. Research suggests a link between smoking, HPV infections, and invasive CC[20]. A comprehensive analysis of numerous studies has demonstrated a significant association between smoking and an elevated risk of CC and CIN score. In trials with a low probability of bias, the overall odds ratio increased to 2.26 from approximately 2.03 overall[21–23]. The present findings indicate that smoking and alcohol consumption, as behavioral habits, are associated with an increased risk of CC and precancerous lesions, primarily in conjunction with HPV infection. The likelihood of developing CC rises with the number of pack-years and the duration of smoking; however, this risk decreases significantly with prolonged cessation, and approximately 15 years after quitting, it reaches a level comparable to that of non-smokers[20]. Alcohol consumption may also increase cancer risk, and according to the WHO, 3.5% of cancer deaths are attributable to long-term alcohol use. Such consumption can contribute to carcinogenesis in the upper aerodigestive tract, liver, colorectum, and breast, although the available data on its association with colorectal cancer remains limited[24]. The impact of smoking and alcohol use on HPV infection and cervical precancerous alterations, which can lead to elevated cell cycle gene expression and histological progression, remains incompletely understood. Therefore, to elucidate the mechanisms and factors contributing to the development of precancerous lesions and CC, further detailed studies are imperative.

While not the primary cause, there is a strong association between HPV infection and CC. Viral oncoproteins are incorporated into the host's DNA, thereby disrupting cellular functions and promoting the uncontrolled proliferation of cells. Persistent HPV has been demonstrated to be a causative agent for CIN. The E6 and E7 proteins have been shown to stimulate proliferation by deactivating key tumor suppressors, such as TP53 and Rb. The G1 phase of the cell cycle is crucial for regulating proliferation; consequently, CDKs are of paramount importance in cancer research[25]. The present study found that *CCNE1* exhibited significantly elevated transcription in patients with high-risk HPV genotypes (e.g., HPV-16 and 18) compared to non-infected individuals. The level of transcription for *CDK2* did not demonstrate statistical significance, although it exhibited a positive trend. Except for the HPV-18 group, a correlation was identified between *CCNE1* expression and persistent infection. For HPV-16 and other high-risk genotypes, a weak yet substantial positive association was identified between *CDK2* and infection persistence. A comparison of the non-infected group with those exhibiting CIN-I and CIN-II/III lesions revealed that their *CCNE1* and *CDK2* levels were significantly lower. According to Christine L. Nguyen and her colleagues, the findings indicate that *CCNE1* and *CDK2* may serve as valuable biomarkers for predicting the risk of CC progression[11]. In their 2019 study, Alla et al. underscored the pivotal role of *CK2α* in HPV-induced carcinogenesis, emphasizing its impact on the transient and stable replication of various HPV strains[26]. According to research findings on the subject, an association has been established between unusual gene expression and tumor growth. It is noteworthy that aberrant cell cycle progression and tumor formation in various malignancies are associated with elevated expression of the *CDK2*[27]. In 2021, Xiaodong Sun and colleagues demonstrated that HPV oncoproteins enhance the expression of *CDK2* and *Cyclin E1*, thereby underscoring the virus's manipulation of host cells. It has been determined that modifying these viral proteins has the potential to mitigate their oncogenic effects and contribute to the prevention of CC progression[28]. A study in 2015 established a correlation between the expression of *CCNE1* and the accelerated proliferation of tumors, as well as the manifestation of aggressive phenotypes in cases of breast cancer[29]. In oral squamous cell carcinoma, Luís Silva Monteiro and his colleagues (2018) found that increased levels of *cyclins A*, *E*, and *B* are correlated with tumor size, lymph node metastasis, histological grade, and clinical stage[30]. Research on various cancer types, including breast and endometrial adenocarcinomas, has demonstrated the critical role of cyclins in regulating the cell cycle and tumor progression. In particular, *CCNE1* is necessary for maintaining genomic integrity and initiating DNA replication. An elevated degree of genomic instability, coupled with an augmented capacity for tumor resistance to therapeutic modalities that target DNA damage, has been observed to be concomitant with its expression[31,32]. Our study suggests a correlation between precancerous cervical lesions and the duration of HPV infection, highlighting increased expression of *CCNE1* and *CDK2*. These findings align with previous research on the roles of these genes in regulating the cell cycle and cancer development. The data enhance our understanding of the elevated expression of *CCNE1* and *CDK2* as mediators of G1/S progression in the cell cycle, in relation to high-risk HPV types and lesion grades. While more research is needed to confirm their clinical significance, *CCNE1* and *CDK2* may serve as promising biomarkers for assessing the risk of cervical lesion progression in HPV infected patients.

The microbiome of the human cervical region could interact with the surrounding microenvironment to preserve tissue balance[33]. Dysbiosis is defined as a disruption in the balance of microbial organisms within the gastrointestinal tract, which can lead to various adverse effects. These effects may include the breakdown of the epithelial barrier, abnormal cell growth, genomic instability, new blood vessel formation, chronic inflammation, and metabolic disturbances[34]. It has been established that persistent inflammation, a consequence of these disruptions, is classified as a carcinogenic factor. This heightened susceptibility to cancer is attributed to the

host's increased vulnerability[35]. An imbalance of bacteria in the cervical region associated with cervicitis is known as bacterial vaginosis (BV). The anti-inflammatory response is reduced by depleting lactic acid. The presence of lactic acid has been shown to have a dual role in regulating inflammation. On the one hand, it has been observed to reduce the inflammatory response triggered by *toll-like receptor (TLR)* activators. On the other hand, lactic acid has been shown to enhance the *IL-1* (interleukin) mechanism by increasing the expression of its antagonist, *IL-1Ra*[36]. It is noteworthy that the environment established by HPV is believed to encourage the continuation of HPV infection, which is a known precursor to CC[37]. Our study revealed that the cervicovaginal microbial composition differed between women infected with HPV and those without infection, although no significant changes were observed in alpha or beta diversity.

Interestingly, *Gardnerella* was depleted in both HPV-positive groups compared to non-infected group, which contrasts with some previous studies. This reduction may be due to changes in the composition of different *Gardnerella* species, as this genus includes pathogenic (*G. vaginalis*, *G. piotii*, *G. leopoldii*) and non-pathogenic (*G. swidsinskii*, *G. pickettii*, *G. greenwoodii*) species, with some non-pathogenic species also present in healthy populations[38]. Notably, both the HPV-positive and non-infected groups were definitively BV-free, indicating that these changes are not attributable to BV but likely reflect microbiome differences in response to HPV infection.

Correlation analysis indicated genotype-specific host–microbiome interactions in HPV-positive individuals, though none of the correlations reached statistical significance (all p-values > 0.05). Based on LDA-based comparative analysis, distinct changes in bacterial genera were observed. In HPV-18, *Nocardia*, *Prevotella*, and *Limosilactobacillus* were increased and showed positive correlations with the expression of either *CCNE1* or *CDK2.* In HPV-16, *Prevotella*, *Actinomyces*, *Corynebacterium*, and *Limosilactobacillus* were increased and also exhibited positive correlations with the expression of one of the *CCNE1* or *CDK2* genes. Additionally, *Bacillus* and *Streptococcus* were reduced in HPV-infected compared to non-infected groups, and this decrease appears to be associated with upregulation of *CCNE1* and *CDK2*. In contrast, in the non-infected group, where these genera were relatively more abundant, gene expression levels were normal or even reduced, supporting their potential role in regulating the cell cycle. Although these associations did not reach statistical significance, the genus-level patterns, together with LDA results, suggest that alterations in specific bacterial populations may influence host cell cycle gene expression and contribute to HPV persistence and CIN progression. To our knowledge, no previous study has simultaneously investigated the relationship between *CCNE1* and *CDK2* expression, HPV genotype, and the cervical microbiome. While this study provides novel insights, the limited sample size and descriptive nature of the findings highlight the need for larger and longitudinal studies to confirm the role of the cervical microbiome in regulating cell cycle gene expression, HPV persistence, and CIN progression.A study conducted by Yuanyue Li found that vaginal microbiome diversity in women with HPV+, CIN-I, CIN-II, CIN-III, and cervical cancer was significantly higher than in the non-infected group (NILM), indicating greater microbial diversity in these groups. At the same time, some indices showed no significant difference. Additionally, analysis of species complexity among the groups revealed considerable differences in microbiome diversity across women with different HPV statuses, CIN grades, and cervical cancer. These findings contrast with our results, as we did not observe significant changes in alpha or beta diversity between HPV-infected and uninfected women. This discrepancy may be attributed to demographic and technical differences between the studies[39]. In our study, a relative depletion of *Lactobacillus* spp. was observed in HPV-18-infected cases, consistent with previous reports linking reduced *Lactobacillus* levels to cervical dysbiosis[40]. Regarding potential pathogens, although some genera, such as *Stenotrophomonas* in the HPV-18 group and *Corynebacterium* in both HPV-16 and HPV-18 groups, showed relative increases compared to non-infected group, these changes did not reach statistical significance. The absence of significant pathogen expansion is likely attributable to the limited sample size. Therefore, while *Lactobacillus* depletion was evident, pathogen overgrowth was not statistically supported in our sample.

In a study conducted by Bing Wei and colleagues, the levels of *Actinobacteria* (primarily consisting of *Gardnerella* and *Atopobium* species) were found to be elevated in individuals with CIN and CC compared to non-infected individuals. In our study, however, no significant increase in these bacteria was observed in the HPV-16 or HPV-18 groups. Instead, Gardnerella showed a decrease (moderate in HPV-16 and severe in HPV-18), and *Atopobium* decreased only in the HPV-18 group. This difference may be due to the fact that our cohort did not include individuals with CC, suggesting that *Actinobacteria* elevation may be associated with more advanced stages of the disease[41]. In the present study, as reported in the results section, specific bacterial genera were found to be increased or decreased in HPV-positive patients based on LDA scores. In HPV-16, *Bifidobacterium* and *Streptococcus* exhibited a trend toward decrease, whereas in HPV-18, *Lactobacillus* showed a pronounced decline, and *Actinomyces* remained nearly unchanged. Although these changes did not reach statistical significance, they represent descriptive trends associated with HPV infection and genotype-specific differences in the cervical microbiome. The reduction of protective bacteria such as *Lactobacillus* may reflect their role in preventing the proliferation of vulnerable cells and maintaining tissue homeostasis. Conversely, increased bacteria, including *Prevotella* and *Streptococcus*, may contribute to chronic cervical inflammation through known mechanisms such as NF-κB pathway activation, increased expression of c-Myc and hTERT, and TLR activation, potentially promoting unchecked cell proliferation, genetic mutations, and immune evasion[42,43]. Although previous studies have indicated that *Prevotella* and *Gardnerella* can act as risk factors for recurrent HPV infections and HSIL, our genus-level data showed a decrease or no significant increase in these bacteria. This discrepancy may be due to the limitations of our study, including the absence of a cohort with invasive cancer, as the rise of these bacteria may be associated with more advanced stages of the disease[43]. Our findings indicate that even in the absence of significant changes in overall microbial diversity, alterations at the genus level may influence the expression of cell cycle regulatory genes and contribute to HPV persistence and CIN progression.

Conclusion

The results of this study indicate that high-risk HPV genotypes, particularly HPV-16 and HPV-18, are associated with increased expression of cell cycle genes *CCNE1* and *CDK2*, as well as specific alterations in the cervical microbiome. Microbial changes included a reduction in protective bacteria such as *Lactobacillus* and an increase in inflammatory or proliferation-associated genera, including *Prevotella*, *Actinomyces*, *Corynebacterium*, and *Limosilactobacillus*. These alterations may create a microenvironment conducive to CIN progression by activating cellular signaling pathways, promoting chronic inflammation, and disrupting cell cycle regulation. Notably, even in the absence of significant changes in overall microbial diversity, genus-level shifts in microbial diversity may interact with host cell cycle gene expression and contribute to the progression of precancerous lesions. The positive correlation between elevated *CCNE1* and *CDK2* expression and high-risk HPV genotypes, as well as the severity of CIN lesions, highlights the potential of these genes and associated microbiome changes as early diagnostic biomarkers for predicting lesion progression in HPV-positive patients. Furthermore, the findings suggest that microbiome modulation via probiotics or other interventions could help restore bacterial balance, reduce inflammation, and improve host responses, potentially lowering the risk of precancerous lesion development. Overall, these results highlight the significance of host–microbiome interactions in cervical lesion development and underscore the need for longitudinal studies with larger cohorts to elucidate the role of the cervical microbiome in regulating cell cycle gene expression and CIN progression. They also provide a foundation for developing targeted preventive and therapeutic strategies.

Study limitations

The limitations of this study include financial constraints, which prevented the analysis of additional samples. It also prevented us from analyzing microbiome profiles in samples from individuals infected with other high-risk HPV genotypes, and there was a lack of statistical analysis to examine the association between HPV infection duration and changes in gene expression or the microbiome. The financial limitation may negatively affect the generalizability and applicability of the findings. Moreover, the absence of analysis regarding infection duration means that no definitive conclusions can be drawn about its impact on gene expression or the microbiome. These microbial changes have the potential to accelerate neoplasia development through persistent inflammation and the persistence of HPV. Therefore, these issues are considered significant limitations of the study, and larger longitudinal studies are recommended to address these questions more accurately. Nonetheless, the findings underscore the critical interplay of viral, molecular, and microbiological factors in CC progression, providing valuable insights for the development of preventive strategies, early detection, and personalized management. In this study, the non-infected group was smaller compared to the HPV-positive group, which may limit the robustness of the statistical comparisons. This limitation was due to ethical constraints and restricted access to HPV-negative biopsies from healthy women. The exclusion criteria were intentionally designed to be stringent, aiming to minimize confounding factors that could influence both microbiome composition and gene expression.

Materials and methods

Study population

This cross-sectional research investigated women referred for cervical screening from April 2024 to March 2025, with samples from patients visiting the women's clinic at Yas Hospital in Tehran, Iran. Initially, all participants completed a questionnaire that included demographic details (age, marital status, smoking habits), recent antibiotic usage in the last two months, presence of autoimmune diseases, and any vaginal medication used in the three days before sampling (The questionnaire has been attached as a supplementary file). All patients had TIN PREP samples taken for HPV testing and pap smears. The experiment was scheduled to start the day after the samples were collected, and they were maintained at 4 °C until then.

Exclusion criteria

Participants were excluded if they were pregnant, menstruating, had immunodeficiency disorders, were on immunosuppressive treatment, or had taken antibiotics in the past 2 to 3 months. Individuals newly diagnosed with bacterial vaginosis were also excluded, as determined by vaginal swab analysis showing reduced *Lactobacillus* species, the presence of clue cells with Gram-negative bacilli, and a fishy odor upon adding 10% potassium hydroxide. Additionally, vaccinated individuals and those using vaginal medications or douching within three days of sample collection were excluded.

DNA extraction

An extraction kit from Favorgen Biotech Corp. was used to extract DNA according to the manufacturer's guidelines (FavorGen, Taiwan). All sample extractions and real-time PCR techniques were performed in a sterile research laboratory. The test was conducted at the Research Centre for Clinical Virology (RCCV) lab at Tehran University of Medical Sciences.

HPV genotyping

The Cobas 4800 HPV Assay was utilized to identify HPV DNA following the guidelines provided by the manufacturer. In conclusion, the Cobas 4800 HPV test is a qualitative in vitro assay designed to identify HPV DNA through automated real-time multiplex PCR. This assay can identify HPV-16, 18, and 12, as well as additional high-risk HPV strains (HPV-31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Additionally, each sample has its *β-globin* internal control[44]. To characterize specific genotypes among the 12 high-risk types identified by the Cobas 4800 HPV assay, we conducted an additional PCR-based genotyping step using the

Genes	Primer sequence (5'–3')	Product size (bp)	Cycling conditions
F: *CCNE1*	GGACACCATGAAGGAGGACG	155	1 cycle of pre-denaturation at 95 °C for 15 min; 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. Melting curve analysis was performed at 55 °C for 15 min under 10 conditions.
R: *CCNE1*	TATTGTCCCAAGGCTGGCTC	155	
F: *CDK2*	AAATCCGCCTGGACACTGAG	192	
R: CDK2	CAGTGAGAGCAGAGGCATCC	192	
F: *ACTB*	CCTTCCTTCCTGGGCATGG	103	
R: *ACTB*	CTGTGTTGGCGTACAGGTCT	103	

Table 5. Nucleotide sequences of the primers used for real-time PCR and amplification conditions.

ROJE Technologies Viga High-Risk HPV Genotyping Kit. This assay allowed for the individual identification of genotypes, such as HPV66, in addition to the pooled cobas results.

Colposcopy and biopsy

After testing positive for high-risk HPV strains, individuals were referred for a colposcopy based on their medical history. Qualified Gynecologists performed all colposcopies under recognized procedures. Lugol's iodine and a 3–5% acetic acid solution were applied to the cervical area throughout the procedure to ensure a thorough examination. Pictures were taken both before and after these treatments were administered. Two biopsy samples were obtained from the affected region if any lesions were detected. One biopsy was fixed in formalin, processed into paraffin blocks, and sent to pathology for histopathological analysis to identify neoplastic or other tissue alterations. The second biopsy was immediately preserved in liquid nitrogen at −70 °C to ensure RNA integrity, enabling high-quality RNA extraction and molecular gene expression analysis. The concurrent use of these two differently preserved samples enabled a comprehensive assessment of histopathology and gene expression, aligning with standard molecular and pathological research practices[45].

RNA extraction

Initially, the biopsy samples were homogenized entirely. Then, RNA was extracted using the Favorgen Biotech Corp. (FavorGen, Taiwan) extraction kit, following the manufacturer's guidelines. All extraction processes were conducted in a sterile environment within a research laboratory, and the samples were subsequently prepared for real-time PCR analysis.

RNA quality

The integrity and purity of every RNA sample extracted were evaluated through absorbance measurements and agarose gel electrophoresis.

cDNA synthesis

Using the SinaClon Iran First Strand cDNA Synthesis Reagent Kit, 1000 ng of RNA was adjusted to produce cDNA.

Real-time PCR

The sequences of the primers produced for the reference gene ACTB and the target genes CDK2 and Cyclin E1 were assessed using the Primer Blast tool. A total of 25 µL was used for each quantitative PCR (qPCR) experiment using SYBR Green PCR Master Mix. To ensure reproducibility, triplicate reactions were performed for each gene, and the absence of contamination was verified using a no-template control (NTC). Using melt curve analysis, the specificity of the amplification was determined. Table 5 displays the complete primer sequences and details the requirements for real-time PCR amplification.

16S rDNA sequencing and sample collection

Biopsy samples from the exocervixes of 21 patients were used in this investigation. Three groups were included in these samples: seven HPV-negative individuals, seven infected individuals with HPV type 16, and seven individuals infected with HPV type 18. After being collected, the specimens were immediately positioned in containers with liquid nitrogen and sent to the microbiological lab at the School of Public Health. Before the DNA was extracted, they were kept at −70 °C.

DNA extraction

DNA was isolated from exocervical biopsy samples according to a specified protocol. First, the biopsy samples were homogenised completely. Lysozyme powder was dissolved in a 10 mM Tris-HCl solution to achieve a final concentration of 10 mg/mL. Five microlitres of this mixture were added, and the biopsy samples were then gently mixed and incubated at 37 °C for fifteen minutes. The High Pure PCR Template Preparation Kit (Roche, Germany) was used for DNA extraction, following the manufacturer's guidelines. The purity and concentration of the DNA were assessed utilizing a NanoDrop One Spectrophotometer. According to the results, the DNA was of high quality and suitable for further applications; the A260/A280 and A260/A230 ratios ranged from 1.8 to 2.1 and 1.9 to 2.1, respectively. After DNA extraction from the biopsy samples, the specimens were carefully preserved and initially sent to Pishgam Company. Subsequently, the samples were transferred to Novogene, a leading company in genome sequencing and advanced microbiology analyses headquartered in Beijing,

China. Novogene conducts extensive microbiome and genomic projects using state-of-the-art next-generation sequencing (NGS) technologies.

Microbiota sequencing data and bioinformatics analysis

Microbiota sequencing data for paired-end reads were analyzed using FLASH (version 1.2.7), and the data's quality was evaluated using QIIME (version 1.7.0). Uparse (v7.0.1001) was used to cluster sequences that showed greater than 97% similarity into OTUs, while the UCHIME algorithm was used to remove chimeric sequences. For taxonomic classification up to the genus level, the RDP classifier was employed. While alpha diversity indices and statistical analyses were conducted using the Shannon Test with QIIME and R, OTUs associated with particular groups were identified using LEfSe analysis[12,41]. Beta diversity was assessed using PCoA and unweighted UniFrac distances to compare microbial community compositions between groups, and PERMANOVA was used for statistical analysis.

Statistical analysis

Data gathering and preliminary preparation were conducted using Microsoft Excel, while the analyses were performed using R software. The graphs were designed using the ggplot2 package. While quantitative variables were summarized using the mean, variance, and median, qualitative aspects were characterized using frequency and percentage. The Kolmogorov-Smirnov test and standard probability Graphs were utilized to assess the normal distribution of quantitative variables.

Non-parametric tests were used to compare two groups and several groups because the distribution of persistence variables and gene expression is not regular. The Mann-Whitney U test was used to compare two groups, while the Kruskal-Wallis test was used to compare multiple groups simultaneously.

Statistical analyses and data visualization were performed using R. ROC curves were generated to evaluate the discriminatory power of *CCNE1* and *CDK2* between HPV-infected and non-infected samples, and AUC values were reported. Additionally, LDA Score analysis was applied to identify key bacterial genera characteristic of each group.

The Spearman rank correlation coefficient was computed and assessed to investigate the relationship between quantitative variables.

Data availability

Due to sensitivity issues, the data used to support the study's conclusions are not publicly available, but upon reasonable request, the corresponding author may provide them.

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Author contributions

The study was designed and conceptualized by R.B. and M.A. The laboratory investigations were done by Z.S., Clinical diagnosis was performed by F.Y., and statistical analysis was carried out by A.R.F. Financial support was provided by R.B. and M.G. The manuscript draft was prepared and written by Z.S., while M.A. performed the final editing.

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Declarations

Competing interests

The authors declare no competing interests

Ethics clearance and participation consent

This study was authorized by the Tehran University of Medical Sciences, Iran's Ethics Committees (IR.TUMS.SPH.REC.1401.073). Every patient who participated in the study signed an informed consent form. The study was conducted in accordance with the principles outlined in the Declaration of Helsinki.

Additional information

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