Shikonin, Gallic Acid, and Hydroxysafflor Yellow A Decrease the Pathogenesis and Normalize the Microbiota of Cervical Erosion

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Abstract

Background and Objective: Cervical erosion is a common gynecological disease that increases the risk of human papillomavirus (HPV) infection and causes cervical lesions. Without prompt treatment, this disease may lead to the occurrence of cervical cancer. According to previous studies, the reproductive tract microbiome is closely related to the occurrence and development of a variety of gynecological diseases, but to date, there have been few studies on the composition of the genital tract flora and the local immune microenvironment of cervical erosion. Currently, the mechanism of interaction between the reproductive tract microbiome and cervical erosion remains unknown.

Materials and Methods: A total of 11 healthy individuals and 15 patients with cervical erosion (including 11 HPV+ patients) were included recruited in this study. Vaginal secretion and cervical mucus were obtained from volunteers before and after treatment. Luminex multi-factor detection technology was used to detect and analyze cytokines, chemokines, and growth factors in the local immune microenvironment of the reproductive tract. A third-generation nanopore high-throughput sequencing platform was used to explore the characteristics of the reproductive tract microbial community structure of HPV+ and HPV− cervical erosion patients. Reproductive tract metabolomics was explored by liquid chromatography-mass spectrometry (LC-MS) for characterization.

Results: Following 21 days of continuous treatment with Jinchuang gel, the smoothness of the cervical surface was restored for all of the 15 patients with cervical erosion, and 4 of the 11 HPV+ patients turned HPV−. After 84 days of continuous treatment, the remaining 7 HPV+ individuals turned negative of HPV−. Furthermore, tThe gel ingredients can improve the local proteolytic environment, reduce the inflammatory response, and promote wound healing. The microbial level of vagina showed that the gel ingredients increased the abundance of *Lactobacillus* and decreased the relative abundance of *Pseudomonas*, *Megasphaera*, and *Hungateiclostridium*, as well as the overall species diversity. Metabolites of Epicatechin, protocatechuic acid, and hydroxybenzoic acid metabolites with activities of anti-bacterial microbes and anti-inflammatory inflammation were effects increased, which subsequently decreased the levels of harmful metabolites (pipecolate, *m*-cresol, 2-hydroxy-2-methylbutyric acid, histamine, and other metabolites). Nicotinic acid and nicotinamide metabolism and the tryptophan metabolism pathway may be related involved to the occurrenceincidence of HPV+ in cervical erosion patients, and niacin and nicotinamide metabolism and the histidine metabolism pathway may be related contributed to the positive outcomes of treatment.

Conclusion: It is speculated that the active ingredients play a role in wound healing, viral clearance, and immune regulation by regulating the activity of the NF-κB and MAPK signaling pathways.

Introduction

There are two types of cervical erosion (CE): pseudo-erosion and pathological inflammatory erosion. Pseudo-cervical erosion[1] usually occurs during puberty and pregnancy[2]. Under the action of estrogen, cervical columnar epithelial hyperplasia and ectropion, also known as cervical columnar epithelial ectopia[3], are normal physiological phenomena. Pathological inflammatory erosion[4] is a common gynecological disease

that consists of chronic cervicitis and local pathological features of CE. Under the influence of some adverse factors [5], inflammation occurs in the genital tract, then caused the surface of the cervical squamous epithelial tissue damage and loss occur and covered by columnar epithelium. The columnar epithelium is weaker and more brittle than the squamous epithelium, and its subcutaneous stroma becomes red with an eroded appearance [6]. These pathological changes disrupt the normal physiology of the cervix while also increasing the risk of human papillomavirus (HPV) infection[7-9].

Traditional Chinese medicine (TCM) consists of unique remedies that are used to prevent and treat diseases[10]. Due to the differences in provenance, natural environment, climate, harvesting season, and processing methods, there are obvious differences in the quality and efficacy of the same medicinal materials from different production areas[11, 12], which has given rise to the concept of “genuine medicinal materials”[13]. Compared to the same herbals in other regions, the so-called genuine medicinal materials refer to those Chinese medicinal materials with high-quality potency selected from a specific region after a long-term TCM practice. The powerful therapeutic effect of Chinese medicinal materials not only depends on the selection of authentic medicinal materials but also benefits from unique processing methods[14]. Excellent Chinese medicinal materials can only be used in medicine after they have been processed to take the essence for further use, discard behind the dregs[15].

The ancient prescription Jin Chuang Yao is a TCM with thousands of years of history that has the functions of purifying wound surfaces, reducing swelling, relieving pain, building muscle, and growing flesh. The ingredients in this formula are (i) zicao/arnebia root (*Radix arnebiae*; *Arnebia euchroma* (Royle) I.M.Johnst.), (ii) safflower flowers (*Carthami flos*; *Carthamus tinctorius* L.), and (iii) Chinese gall (*Galla chinensis* (Wu Bei Zi); gallnut of Chinese sumac). The active ingredients of zicao root, safflower, and Chinese gall are shikonin, hydroxysafflor yellow A, and gallic acid, respectively. Shikonin exhibits numerous biological properties, such as antioxidant[16], wound healing [17], and antibacterial[18] properties, among others. Safflower flowers contain hydroxysafflor yellow A, kaempferol, and other active ingredients. Hydroxysafflor yellow A plays an important role in anti-cerebral thrombosis and has anti-tumor[19-21] and wound tissue edema-reducing functions[22]. Gallic acid, the active ingredient in *Galla chinensis*, is a metabolite of trihydroxy benzoic acid. It exhibits strong anti-oxidant [23, 24], anti-inflammatory[25], and anti-cancer[26] activities and can protect biological cells, tissues, and organs from oxidative stress damage[27].

Cervical erosion is a common gynecological disease that is a specific manifestation of inflammation of the female reproductive tract. The onset age of cervical erosion is tending younger, especially in women of childbearing age. Early wet microscopic examination or culture-dependent methods suggest that CE is related to genital tract flora and local inflammatory factors[28]. Concomitant with CE, it was observed that the detection rate of *Lactobacillus* significantly decreased, and local cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-8 increased[29]. To date, there have been few studies on the composition of the reproductive tract microbiome and the local immune microenvironment that exists when CE is present. The interaction mechanism between reproductive tract microbiota and CE remains unknown.

With the rapid development of sequencing technology and bioinformatics, there has been an increase in the number of studies showing the close relationship between the imbalance of the reproductive tract microbiota and the occurrence and development of many gynecological diseases [30-32]. It is necessary and important

to study and analyze the changes in the vaginal microbiota, at the local immune and metabolic levels, during CE so as to explore its pathogenesis, prevention, and treatment.

Materials And Methods

Jinchuang gel formula

In this study, the gel was prepared according to the main components and proportions of the ancient prescription Jin Chuang Yao. The gel mainly contains 99.99 % of shikonin, hydroxysafflor yellow A, and gallic acid, which were obtained from the extracts of *Radix arnebiae*, *Carthami flos*, and *Galla chinensis*, respectively, and prepared in a fixed ratio of 1:1:1 (Gel registration certificate No.: Luxie injection permit 20182660140).

Study population recruitment

The study participants were patients who visited the obstetrics and gynecology OPD (outpatient department) at Xin Kaiyuan Hospital in Xiamen from October 2020 to February 2021, who fulfilled the inclusion criteria and provided valid consent. The recruitment of the study population was reviewed and approved by the Medical Ethics Committee of Xin Kaiyuan Hospital in Xiamen (Approval Document No.: XMXKYMHIRB-2020-09-R1).

Inclusion criteria

Patients diagnosed with CE were included if they met the corresponding clinical diagnostic criteria, volunteered to participate in the experiment, and signed the informed consent form.

Exclusion criteria

Pregnant women and women who had given birth in the preceding 6 weeks; patients on oral contraceptive pills; patients with known cervical intraepithelial neoplasia (CIN) or who had been treated for CIN or cervical cancer; patients who had used vaginal medication within the preceding 2 weeks, had a vaginal lavage within the preceding week, and had engaged in sexual intercourse within the previous 48 h; and women with a long history of heavy alcohol consumption and smoking were excluded from this study.

Specimen collection and HPV genotyping

The enrolled patients underwent colposcopy after treatment for 0, 7, and 21 days with the gel. Before administration of the treatment on the 1^{st}, 7^{th}, and 21^{st} days, cervical and vaginal secretions were extracted with Merocel^{TM} cervical sponges (Medtronic Xomed, Jacksonville, FL, USA) and cotton swabs. Cervical secretions were used for HPV typing, and vaginal secretions were used for cytokine detection and vaginal microflora sequencing. In the healthy control (HC) group, there was no CE disease, and these patients did not receive the gel. The swab samples for each participant were collected at the hospital and were immediately stored at −80°C until analysis. HPV genotyping of cervical specimens was conducted for 19 HPV types

(HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 82, 6, and 11) using the HPV nucleic acid detection and genotyping kit (AmoyDx) according to the manufacturer's instructions.

Protein extraction from vaginal swabs

Proteins for cytokine analysis were extracted from cotton swabs using a previously established protocol[33]. First, the wet weight of each swab was recorded. Each swab was then placed in a 2-mL Spin-X centrifuge filter tube (Corning, Costar, USA), and 1 mL of extraction buffer [phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA), 256 mM NaCl, and 100 μg/mL aprotinin (Wako, Amagasaki, Japan)] was slowly added. The swabs were incubated at 4°C for 2 h and then centrifuged at 15,000 rpm for 15 min at 4°C. Next, the filter membrane was rinsed with 500 μL eluent several times, and the bacteria were collected on the filter membrane. The extracts for cytokine and metabolome analysis were partitioned and frozen at −80°C until further testing. The bacterial eluents were immediately used for bacterial genome extraction.

Immune cytokine assays

We performed Luminex-based Milliplex MAP (Millipore, Billerica, MA, USA) multiplex assays on all the participants' vaginal secretions. We quantitated 27 immune cytokines, namely, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor basic FGF , vascular endothelial growth factor(VEGF), interferon-inducible protein(IP)-10, eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor(G-CSF), Interferon (IFN)-γ, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-β, platelet-derived growth factor (PDGF)-BB, regulated upon activation normal T cell expressed and secreted factor (RANTES), and TNF-α, following the company's kit procedures (Human Cytokine 27-Plex Panel Kit, Bio-Rad). The results were statistically analyzed via GraphPad Prism software (*t* test)[34].

DNA isolation and full-length 16S rRNA gene sequencing

In total, DNA was extracted from one swab from each sample using the QIAamp® DNA Investigator kit (Qiagen, USA) as described by the manufacturer's instructions and used as a PCR template. The concentration and purity of the extracted bacterial DNA were determined using the Qubit 3.0 Fluorometer (Thermo Scientific, USA) and NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). PCR amplification of 16S rRNA genes was conducted using the 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK) and LongAMP Taq 2x Master Mix (New England Biolabs, Ipswich, MA, USA). PCR products were purified using VAHTS DNA Clean Beads (Vazyme) and quantified using a NanoDrop spectrophotometer and Qubit 3.0 fluorometer. According to the manufacturer's instructions, a total of 100 ng DNA was used for library preparation, and MinION sequencing was performed using R9.4 flow cells (FLO-MIN106; Oxford Nanopore Technologies). MINKNOW software ver. 1.11.5 (Oxford Nanopore Technologies) was used for data acquisition.

16S rRNA-based vaginal community analysis

First, the FAST5 files obtained from sequencing were transformed into FASTQ files using Guppy (version 4.0.14) software. Second, EPI2ME software was used to split barcodes. The quality control and de-coupling

of the FASTQ files were performed using NanoPlot (version1.30.1) software. Finally, nanoFILT (version 1.8.0) software was used to filter the data. Sequences were filtered based on length, quality value (Q value), and numbers of reads, with retention of only sequences > 1.2 Kb and < 1.6 Kb, with a Q-value ≥ 11, and numbers> 15,000 reads. Some sequences with too short lengthsand low sequencing quality were filtered. After referring to the RefSeq database of the NCBI species classification, Centrifuge software (version 1.0.4) was used to conduct species composition classification. Based on the annotation results, the alpha diversity index was calculated using the R language Vegan package. Megan software was used to visualize the species classification results. Stamp software was used to group and compare the multiple samples of a single sequence to conduct sample difference analysis.

Sample preparation for metabolomics

We included a total of three HC samples and four $CE^{+}HPV^{+}$ samples, and the vaginal secretions from the HC group and the $CE^{+}HPV^{+}$ group after treatment for 0 and 21 days were employed for metabolome analysis. Metabolomic analysis was performed starting with 250 μL of the extracts added to 1,000 μL MeOH/ acetonitrile (ACN) (v:v, 1:1), which was vortexed for 30 s and then sonicated for 10 min in a 4°C water bath. The samples were then incubated for 1h at −20°C to facilitate protein precipitation and centrifuged for 15 min at 13,000 rpm and 4°C. Finally, the supernatant was subjected to non-targeted liquid chromatography (LC) mass spectrometry (MS)-based metabolomics analysis (Core Facility of Biomedicine, Xiamen University, China). LC-MS was performed using ultra-performance liquid chromatography (UPLC) with a Triple ToF 5600+ (AB Sciex, USA) high resolution/sensitivity mass spectrometer. The samples were run in positive- and negative-ion modes. The raw data were pre-processed, identified using a metabolite database, and then analyzed.

Metabolite pre-processing and statistical analysis

Metabolites were excluded from the analysis if they were undetected in ≥ 90% of each biological group. We assumed that the remaining missing values were caused by an abundance of metabolites below the detection limit. The missing variables were imputed using LoDs (1/5 of the min positive value for each variable) obtained for a given metabolite. Subsequently, data normalization was performed to meet the subsequent statistical analysis requirements. Important features were selected from a volcano plot with a fold-change threshold (x) of 2 and a *t* test threshold (y) of 0.05 by univariate analysis. Orthogonal partial least squares discrimination analysis (OPLS-DA) was used to analyze the changes in metabolic patterns in different biological groups. Score plots of variable importance in projection (VIP) were used to evaluate the discriminatory metabolites.

A filter VIP > 1.5 identified potential differential metabolites. It served as a potential biomarker if P < 0.05, FC > 2 or FC < 1/2, or VIP > 1.5. The relevant metabolic pathways were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with MetaboAnalyst 5.0 (www.metaboanalyst.ca). In addition, volcano plots, OPLS-DA score plots, and VIP score plots were also generated using the open-source software MetaboAnalyst 5.0 (www.metaboanalyst.ca). We examined how differential flora obtained by 16S sequencing related to the differential metabolites obtained by LC-MS using Spearman's correlation carried out by the psych, reshape2, and heatmap packages of R software.

Results

Clinical characteristics of the recruited subjects

A total of 15 diseased patients (DIS) and 11 healthy controls (HC) were recruited. Among the 15 patients in the disease group, 4 exhibited only CE, and 11 were HPV+ with CE. Age was represented by the mean ± standard error, and there was no significant difference in age among the groups (Table 1). After 21 days of treatment, the smoothness of the cervical surface of the 15 patients with CE was restored (Figure 1), and testing showed that 4 of the 11 HPV+ patients became HPV−. After 84 days of treatment, the status of the remaining 7 HPV+ patients changed to HPV−.

Cytokine measurements

Measurement of 27 cytokines in the vaginal microenvironment was performed using Luminex assays (Figure 2). The expression levels of IL-1β, eotaxin, G-CSF, MCP-1, and IL-4 were significantly different between the HC and DIS groups. Except for IL-1β, the other four cytokines showed low expression levels. IFN-γ and IL-1ra were significantly different between the CE^+ and CE^+HPV^+ groups, and IFN-γ, IL-1ra, and basic FGF were expressed at low levels in the 7D/21D-CE^+HPV^+ group, suggesting that the HPV virus may affect the vaginal immune microenvironment of patients with CE and aggravate the inflammatory response of chronic wounds. The cytokine eotaxin was significantly changed in the CE^+ group, while the cytokines IL-6, IFN-γ, IL-1ra, eotaxin, basic FGF, and PDGF-BB were significantly changed in the CE^+HPV^+ group, suggesting that these cytokines may be related to the disease. After treatment with the gel, the environment of protein hydrolysis related to local inflammation of the reproductive tract was improved. The aggregation of cytokines to the site of inflammation plays a biological role in promoting wound healing and virus clearance.

Taxonomic assignment of 16S rRNA gene amplicon sequences

In this study, 33 samples of genital tract secretions were included to study the differences in the vaginal microbial community structure. A total of 1,392,019 sequences were obtained from the 33 samples, with an average of 42,182 sequences per sample.

Alpha diversity analysis

All the alpha diversity indices are presented in Table 2. The α diversity index of the HC group was lower than that of the other groups, which indicated that the composition diversity of the reproductive tract microflora in the healthy group was lower than of the other groups. There was high diversity ($P < 0.001$) in the DIS group, and there was no significant difference in the diversity or richness of the bacteria in the genital tract between HPV+ and HPV− patients. There was a significant reduction in the genital tract flora diversity and richness over time and an overall improvement in health.

Analysis of the vaginal microbial composition

The vaginal microbiota of healthy females is mainly dominated by *L. crispatus* in Firmicutes, which were found to have the highest relative abundance, followed by *Lactobacillus C25* and *L. iners* with low diversity,

and this is consistent with other reports. The structures of the vaginal microbiota in females in the DIS and HC groups were significantly different (Figure 3), with high diversity and low relative abundance of *Lactobacillus*, while the relative abundance of *Pseudomonas*, *Hungateiclostridium*, *Thermoclostridium*, and other genera was significantly increased in DIS group. The relative abundance of *Pseudomonas* and *L. iners* was higher in the CE^+HPV^+ group than in the CE^+ group (Figure 4). Studies have shown that high-risk HPV infection is positively correlated with *Pseudomonas*, and therefore, a high abundance of *Pseudomonas* is closely related to the presence of the HPV virus in the CE^+HPV^+ group. A high abundance of *L. iners* can stimulate a local immune response, which may assist in eliminating the HPV virus.

After 21 days of the gel treatment, the diversity of the microbial community significantly decreased, with the relative abundance of several possible pathogens significantly decreasing in the CE^+ and CE^+HPV^+ group. However, there was no significant change in the relative abundance of *L. crispatus* in the treatment group, which showed that the relative abundance of *L. iners* significantly increased and was higher than that of the HC group. The relative abundance of *L. jensenii* mainly increased in the HPV− patients whose status changed from HPV+ to HPV−, but this trend was not observed in the CE^+ group before and after treatment, which may indicate that *L. jensenii* has a positive effect on the elimination of the HPV virus. In addition, the presence of the HPV virus did not alter the effectiveness of the gel in the treatment of CE or the overall recovery toward the beneficial microbial structure.

Principal component analysis (PCA)

The microbiome composition in the HC group was similar between each sample (Figure 5), and the DIS group deviated from the HC group, showing that the microbiome composition in the DIS group was significantly different from that of the HC group. Partial samples between the CE^+HPV^+ and CE^+ groups got together, and this may be due to CE patients at the early stage of HPV infection, during which the effect of the HPV virus on the genital tract microbial structure is weak or it could also suggest that the genital tract of CE+ patients are more easily infected with HPV compared to that of CE− patients.

As the treatment continued, part of the samples tends to get together with the HC group in CE^+ and CE^+HPV^+ groups, and the relative distance shortened obviously. These results indicated that the composition of the reproductive tract microbial community of these samples was similar to that of the HC group at the genus level after treatment. There was significant deviation in a portion of the samples before and after treatment, with a significant trend toward normalization. These aspects of microbial community structure illustrate that this portion of patients were cured due to the efficacy of the gel.

Discriminatory microbiota analysis

At the species level, *Lactobacillus* was highly expressed in the healthy group (Figure 6), while *Bacillus*, *Anaerostipes hadrus*, and *Clostridium* sp.*CT4* were more abundant in the CE^+ group. *Bacillus* sp. was more abundant in the CE^+HPV^+ group, suggesting that these bacteria may be involved in the occurrence of CE. After 21 days of treatment, the relative abundance of *Lactobacillus* increased, but the microbial structure of the reproductive tract was not fully recovered. Overall, the flora of these patients changed so that it developed

toward that of healthy individuals, indicating the importance of *Lactobacillus* in maintaining the health of the female reproductive tract.

Metabolomics analysis of the vaginal microenvironment

In this study, 301 metabolites were obtained by running in positive and negative ion patterns via UPLC-MS according to fold change (FC > 2 or FC < 1/2) and *t* test P values (P < 0.05) (Figure 7). OPLS-DA was used to filter unrelated signals. Metabolites with VIP ≥ 1.5 were selected as biologically significant differential metabolites between groups (Figure 8). Several significant characteristic metabolites were observed to distinguish each biological group.

The distance between the samples of the HC and CE^+HPV^+, CE^+HPV^+, and 21D-CE^+HPV^+ groups was relatively large, and the metabolic pattern was significantly separated. Compared with the HC group, the metabolites with significantly high expression in the CE^+HPV^+ group were pipecolate, *m*-cresol, Dl-threitol, and 2-hydroxy-2-methylbutyric acid. Pipecolate is involved in lysine metabolic pathways, and previous studies have shown that it can change oxidative stress to increase the survival of cells exposed to H_2O_2 under duress. A higher concentration of pipecolate in the CE^+HPV^+ group may increase the resistance of pathogenic bacteria to H_2O_2, lead to long-term colonization of pathogenic bacteria in the reproductive tract and is a factor that impedes genital tract health recovery. *m*-Cresol has been shown to cause papilloma in cancer studies, suggesting that it may be an abnormal metabolite associated with the HPV virus. 2-Hydroxy-2-methylbutyric acid is commonly found in the urine of hydroxyglutaric aciduria and diabetes patients. High concentrations of 2-hydroxy-2-methyl butyric acid suggest that it may be associated with the HPV virus and CE. In the CE^+HPV^+ group, there was significant underexpression of cholic acid, stachyose β-nicotinamide adenine dinucleotide, indolelactic acid, L-tryptophan, urate, and adenosine, which may be beneficial metabolites associated with maintaining reproductive tract health.

Compared with the CE^+HPV^+ group, epicatechin, protocatechuic acid, and x-dihydroxybenzoate metabolites with antibacterial and anti-inflammatory effects were significantly overexpressed in the 21D-CE^+HPV^+ group, which are metabolites beneficial for reproductive tract health. Histamine, acetoin, butanoate, and isobutyrate were significantly underexpressed in the 21D-CE^+HPV^+ group. Histamine may be related to the antimicrobial actions of overcoming the obstacles of an acidic environment, protecting pathogens against lactic acid, and the influencing of host immune defense.

The metabolic pathway correlation analysis was performed on the identified differential metabolites, and the pathways were elucidated according to the impact value > 0.1 (Figure 9). The results showed that there were two disease-related metabolic pathways involved between the HC and the CE^+HPV^+ groups, namely nicotinate and nicotinamide metabolism and tryptophan metabolism. There were two metabolic pathways related to the treatment between the CE^+HPV^+ and 21D-CE^+HPV^+ groups, namely nicotinate and nicotinamide metabolism and histidine metabolism.

Correlation analysis between the vaginal microbiota and metabolome

Spearman correlation analysis was conducted to analyze how HPV and CE associated metabolites related to HPV and CE associated bacterial species in the reproductive tract (Figure 10). The results showed that the relative abundance of *Lactobacillus* in the HC group was correlated with the concentration of beneficial metabolites such as stachyose, adenosine, L-tryptophan, and β-nicotinamide adenine dinucleotide, which jointly maintain the health of the reproductive tract. There was a high concentration of *m*-cresol and Dl-threitol in the $CE^{+}HPV^{+}$ group, which with *Bacillus* may promote the occurrence and development of CE. Twenty-one days after treatment with the gel, the vaginal flora were obviously normalized; *Lactobacillus* and the concentrations of protocatechuic acid, epicatechin, and x-dihydroxybenzoate metabolites were significantly positively related, while histamine, acetoin, butanoate, and isobutyrate were negatively correlated, suggesting that *Lactobacillus* probiotics and beneficial metabolites with anti-bacterial and anti-inflammatory properties promote each other and jointly regulate the homeostasis of the reproductive tract microenvironment.

Based on the known possible molecular mechanisms and the main signaling pathways of these biological effects, and based on the results of this study and the KEGG pathway analysis, it is speculated that the Jinchuang gel plays a role in virus clearance and immune regulation in wound healing by regulating the activity of the NF-κB and MAPK signaling pathways (Figure 11).

Discussion

In this study, when the reproductive tract was in the diseased state, local cytokines were at a low level. This may be related to the proteolytic environment formed by inflammatory cells infiltrating the wound surface, which would degrade and isolate various growth factors and cytokines on the wound surface and make it difficult for them to function normally[35]. It also may be closely related to the immunosuppression associated with persistent HPV infection. HPV virus infected cells may suppress the immune response by inhibiting one or more important signals[36].

IFN-γ and IL-1ra levels were low in the vaginal microenvironment of $HPV^{+}CE^{+}$ patients, which may be related to persistent HPV infection caused by suppression of the body's immunity by HPV, or it could be that the level had not recovered due to IFN-γ clearance the HPV virus. HPV infection can stimulate the production of IFN-γ and thus antagonize the carcinogenic factors associated with high-risk HPV[37], and conversely, low IFN-γ secretion levels may be associated with persistent HPV infection and the development of HPV–associated tumors. In addition, the aggravation of inflammation caused by HPV may promote the development of tumors, tumor cell survival, and angiogenesis[38].

The Jinchuang gel contains shikonin, hydroxyl safflower yellow A, and gallic acid, which confer anti-inflammatory, immunomodulatory, and antiviral properties. These gel components can regulate the NF-KB and MAPK signaling pathways to alter the local lesion’s chronic inflammatory environment with cytokine involvement so as to enhance the recovery of the cervical surface. At the same time through induced cell apoptosis[39], which includes HPV genome, regulating immune microenvironment to reduce the viral load.

The diversity of the vaginal microbiome in the healthy group was low, and the vaginal microbiome was mainly composed of *L. crispatus*, which is the most commonly produced H_2O_2 lactobacillus in the vagina,

followed by *L. jensenii*. *Lactobacillus* resist the invasion of pathogens by producing H_2O_2, lactic acid, and bacteriocins, which play an important role in maintaining the health of the reproductive tract. The diseased group exhibited a high diversity of bacteria and a low relative abundance of *Lactobacillus*, which was consistent with other reports[40]. The relative abundance of *L. iners* in the genital tract of HPV+CE+ patients were higher than that in the genital tract of CE+ patients. Studies have shown that *L. iners* has a higher relative abundance in the female genital tract of HPV+ women[41, 42], which has the ability to stimulate a local immune response and may contribute to the elimination of the HPV virus[43].

After treatment with the gel, although the disease symptoms were ameliorated, the vaginal microbial structure was still suboptimal, and the reproductive tract pH value was higher than healthy women, which may be unsuitable for the survival and function of *L. crispatus* in the vaginal microenvironment. Therefore, the relative abundance of *L. crispatus* did not significantly increase, while *L. iners* exhibited strong environmental adaptability[44]. The increase in the abundance of *L. iners* contributed to the restoration of the normal reproductive tract microbial environment, which was consistent with other research results[43]. *L. jensenii* has a strong capacity to produce lactic acid and H_2O_2. In addition, *L. jensenii* can induce human peripheral blood mononuclear cells to produce IFN-γ, and therefore, the abundance of *L. jensenii* increased in individuals who changed their status from HPV+ to HPV-, which assisted in immune cells clearing the HPV virus, bypassing virus escape, and restoring the immune microenvironment to the steady state [45].

In the HC group, there was a strong positive correlation between adenosine, stachyose, and the relative abundance of *Lactobacillus*, which jointly promoted the health of the female reproductive tract. Stachyose is a non-reducing functional oligosaccharide that can promote the proliferation of beneficial bacteria such as *Lactobacillus*, regulate immunity, and inhibit the growth of harmful bacteria, thus improving the local microbiome[46]. Both adenosine and β-nicotinamide adenine dinucleotide confer anti-inflammatory effects[47, 48]. Adenosine can inhibit phagocytic adhesion and toxic oxygen metabolite production, which is a metabolite related to reproductive tract health.

There was a strong negative correlation between high concentrations of histamine, pipecolate, 2-hydroxy-2-methylbutyric acid, *m*-cresol, and DI-threitol, and *Lactobacillus* in HPV^+CE^+ patients. Histamine may be related to bacteria overcoming the obstacle of an acidic environment[49], protecting pathogens against lactic acid, and influencing the host immune defense[50, 51]. These possible metabolic biomarkers of HPV may contribute to infection with sexually transmitted viruses by co-acting with certain pathogens, such as *Pseudomonas*. Because HPV^+CE^- individuals were not included in this study and the sample size was small, these possible metabolite biomarkers need to be verified by subsequent expansion of the sample size and identification and adjustment of other potential confounding variables.

Conclusions

The Jinchuang gel exhibited significant efficacy in the treatment of pathological CE and elimination of the HPV virus by regulating the activity of the NF-κB and MAPK signaling pathways and thus regulating the reproductive tract immune microenvironment. At the microbial level, increasing the abundance of probiotics such as *Lactobacillus* decreased the diversity of pathogenic bacteria and species, thus improving the

microbial community structure of the reproductive tract. In terms of reproductive tract metabolism, increased secretion of anti-bacterial and anti-inflammatory metabolites reduced the levels of harmful metabolites and promoted reproductive tract health recovery.

The innovation of this research lies in the use of Jinchuang TCM gel, with the main ingredients being zicao root, safflower flowers, and Chinese gall. Advanced nanopore sequencing technology was used to explore the genital tract microbial community structure in patients with pathological CE with or without HPV and to analyze the local immune microenvironment and metabolite levels to elucidate the cause and thus the most effective treatment for CE.

A potential limitation of this study is that the sample size is relatively small, and the results may be one-sided. In the future, it will be necessary to record in greater detail various other individual data that may affect the experimental results as the source of heterogeneity of study participants. $HPV^{+}CE^{-}$ individuals were compared with $HPV^{+}CE^{+}$ individuals and analyzed, and soluble, volatile, and non-volatile metabolites were analyzed by GC-MS and LC-MS, so as to achieve a more comprehensive characterization of reproductive tract metabolites. An increase in the clinical sample size would increase our understanding of CE and different HPV types (high-risk or low-risk HPV) and enable analysis of different severities of CE at the vaginal microbial, immune microenvironment, and metabolic levels.

There is a complex relationship between diseases and the microbiome. The microbiota can interact with tissues through their own constituent products and metabolites, thus promoting the occurrence and development of diseases. Similarly, some local inflammatory reactions caused by diseases can interfere with the dynamic balance between the microbiome constituents. Additional experiments must be performed to study the correlation between reproductive tract microbiota and the occurrence and development of gynecological diseases. It is believed that with the continuous development of microbiome, metabolomics, proteomics, transcriptome, and other omics fields, we will have a clearer understanding of the intricate relationship among them.

Declarations

Ethical Approval and Consent to Participants: Not applicable.

Consent for publication: Those participating authors has a verified history of publications using the personal and academic email address and trackable internet profile(s) for correspondence and authorship.

Availability of data and materials: Applicable for all scientists for sure.

Competing Interests: We ensure that the authors do not have competing interests at all.

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Author Contribution: ZQH carried out total RNA extraction and PCR detection, participated in miRNA expression analyses, bioinformatics analyses and article drafting, and revised the manuscript. SC and LS confirmed the target of miRNA through western blot. KY performed TaqMan-qPCR to validate the identified miRNA. QC and ZL polished the tables and figures. QL and QM performed the clinical evaluation and the assessment of CE by endoscopy. CMT, QM, and QL were responsible for the experimental design and data interpretation.

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Author's information: All authors are trackable scientists (QHZ and XTW are working on Vaginal -Omics and Microbiome studies), medical doctors (YCB are fully dedicating on cancer prevention and rehabilitation) and professors (CJC, HCL and CMT are focusing on translation medicine of microbiota, biomarker deciphering and cancer management) in public domain and institute profiles.

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Tables

Table 1. Clinical characteristics of the recruited subjects. Cervical erosion phase 1: erosion area did not exceed 1/3 of the entire cervical area; cervical erosion phase II: erosion area accounts for 1/3–2/3 of the entire cervical area; cervical erosion phase III: erosion area accounts for more than 2/3 of the entire cervical area.

Clinical characteristics	Health groups (N=11)	Disease group (N=15)	7 days of treatment	21 days of treatment	84 days of treatment	P value
Age (Mean ± SEM)	45.36±1.998	44.33±2.233	44.33±2.233	44.33±2.233	44.33±2.233	0.7440
Erosion in installment	–	Phase I N=11 Phase II N=3 Phase III N=1	Phase I → Health (N=11) Phase II → Health N=2 Phase III → Phase I (N=1) Phase III → Phase II (N=1)	All recovered N=15		—
HPV infection	HPV-(N=11)	HPV+(N=11) HPV-(N=4)	–	HPV+ →HPV- (N=4)	HPV+ →HPV- (N=11)	—

Table 2. Alpha diversity analysis. The *t* test was used to analyze the significant differences between the two biological groups. Compared with the healthy control (HC) group: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05: compared with the diseased patients(DIS) group: △△P < 0.01, △P < 0.05.

Groups	Shannon-wiener	Gini-simpson	Chao1	ACE
HC	0.822± 0.05248	0.3480 ± 0.04852	264.5 ± 44.75	242.0 ± 48.18
DIS	3.786 ± 0.2871 ****	0.8900 ± 0.05327 ****	1112 ± 88.49 ****	1147 ± 104.4 ****
7D	2.487 ± 0.4649 *	0.5792 ± 0.09629 △	947.4 ± 144.4 **	976.7 ± 144.9 **
21D	1.952 ± 0.4377 △△	0.5000 ± 0.1020 △△	893.2 ± 141.9 **	913.7 ± 147.4 **
CE^+	3.983 ± 0.04978 ****	0.9400 ± 0.02082 ***	1079 ± 185.5 **	1130 ± 222.7 **
7D-CE^+	3.330 ± 0.7477 **	0.7425 ± 0.1409 *	1111 ± 221.6 **	1123 ± 205.6 **
21D-CE^+	2.050 ± 0.9398	0.4933 ± 0.2245	867.6 ± 185.6 **	908.8 ± 192.5 **
CE^+HPV^+	3.638 ± 0.5198 ***	0.8525 ± 0.09286 **	1137 ± 98.64 ****	1160 ± 114.8 ****
7D-CE^+HPV^+	2.065 ± 0.5599	0.4975 ± 0.1214	865.4 ± 189.1	903.5 ± 195.3
21D-CE^+HPV^+	1.903 ± 0.5307	0.5033 ± 0.1220	906.0 ± 203.3 *	916.1 ± 211.6 *

Figures

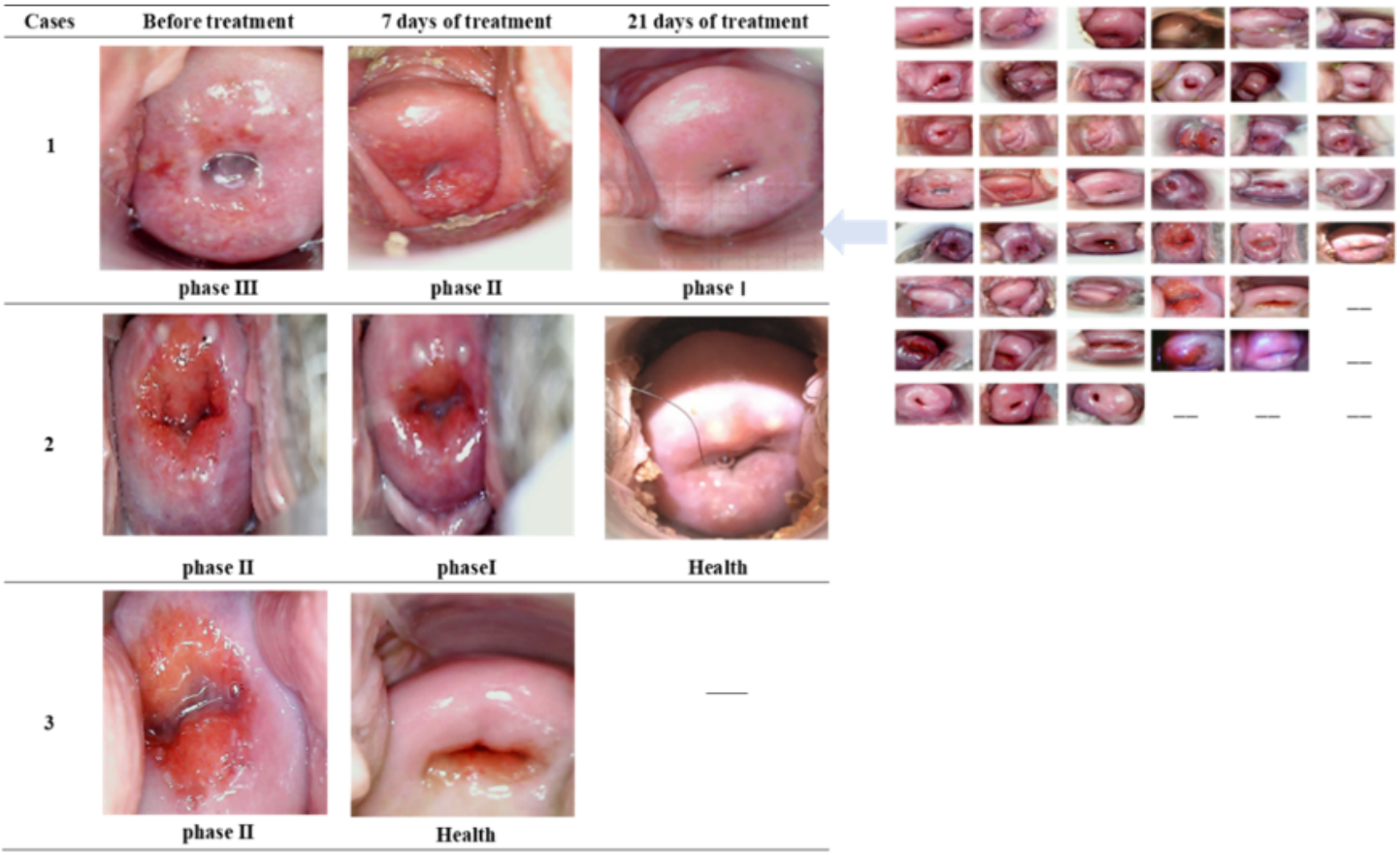

Figure 1

Colposcopy appearance of the cervix in three cases of cervical erosion.

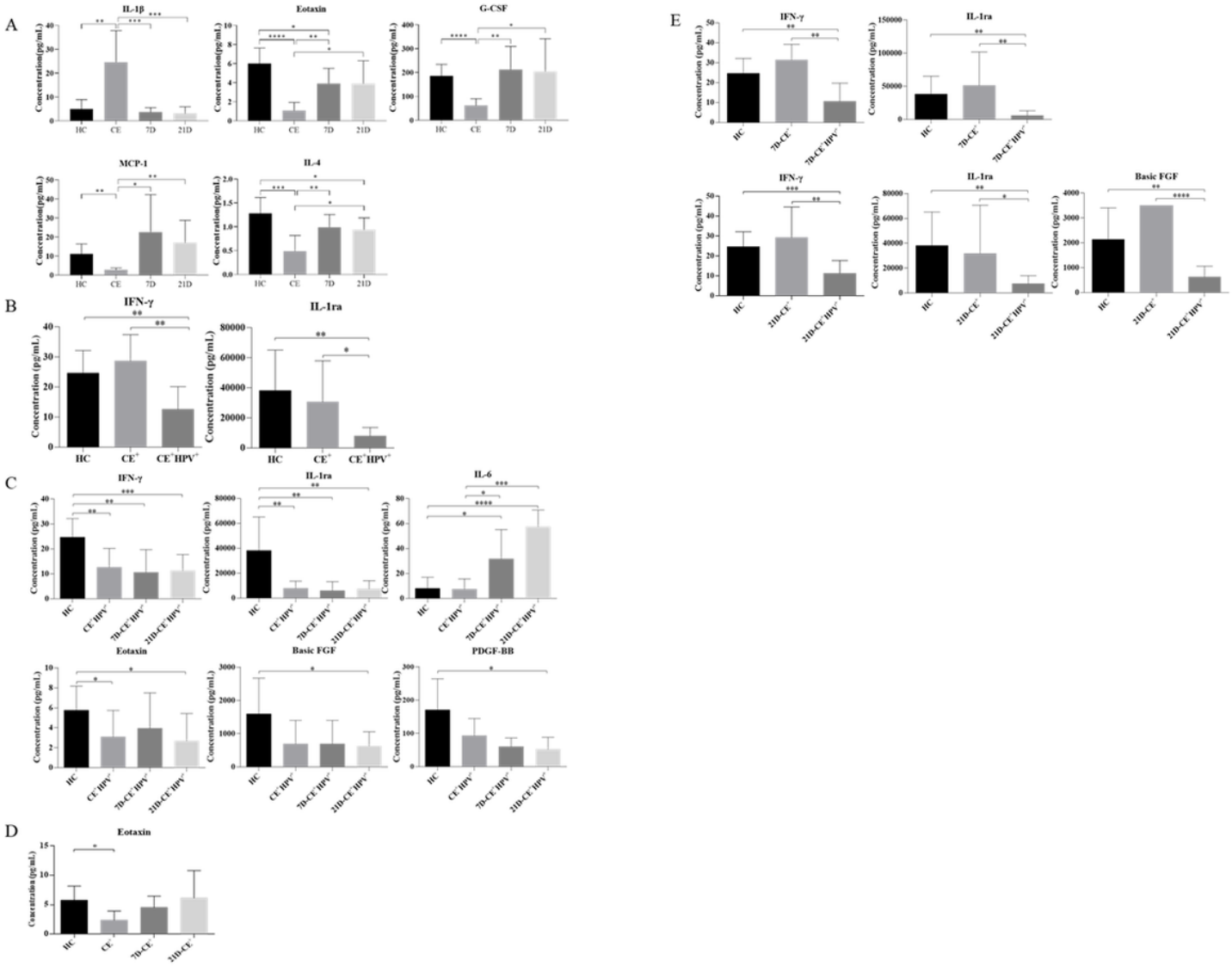

Figure 2

Cytokines and chemokines whose concentration significantly changed during the study period (P < 0.05). HC, healthy control group; DIS, diseased group; CE^+, cervical erosion group; CE^+HPV^+, cervical erosion with HPV+ group; 7D, DIS patients treated for 7 days with the gel. 21D, DIS patients treated for 21 days with the gel. 7D-CE^+, CE^+ patients treated for 7 days with the gel. 21D-CE^+, CE^+ patients treated for 21 days with the gel. 7D-CE^+HPV^+, CE^+HPV^+ patients treated for 7 days with the gel. 21D-CE^+HPV^+, CE^+HPV^+ patients treated for 21 days with the gel. Cytokine or chemokine names are reported in the title. The data are expressed as concentration (pg/mL) on the *y*-axis. The diagrams show the mean values, with error bars representing the standard deviations. The differences were calculated by *t* test (*p < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001). A: The cytokine levels between the HC, DIS, 7D, and 21D groups. B: The cytokine levels between the HC, CE+, and CE^+HPV^+ groups. C: The cytokine levels in the CE^+HPV^+ group after 0, 7, and 21 days of treatment. D: The cytokine levels in the CE^+ group after 0, 7, and 21 days of treatment. E: The cytokine levels in the CE^+ and CE^+HPV^+ groups after 7 and 21 days of treatment.

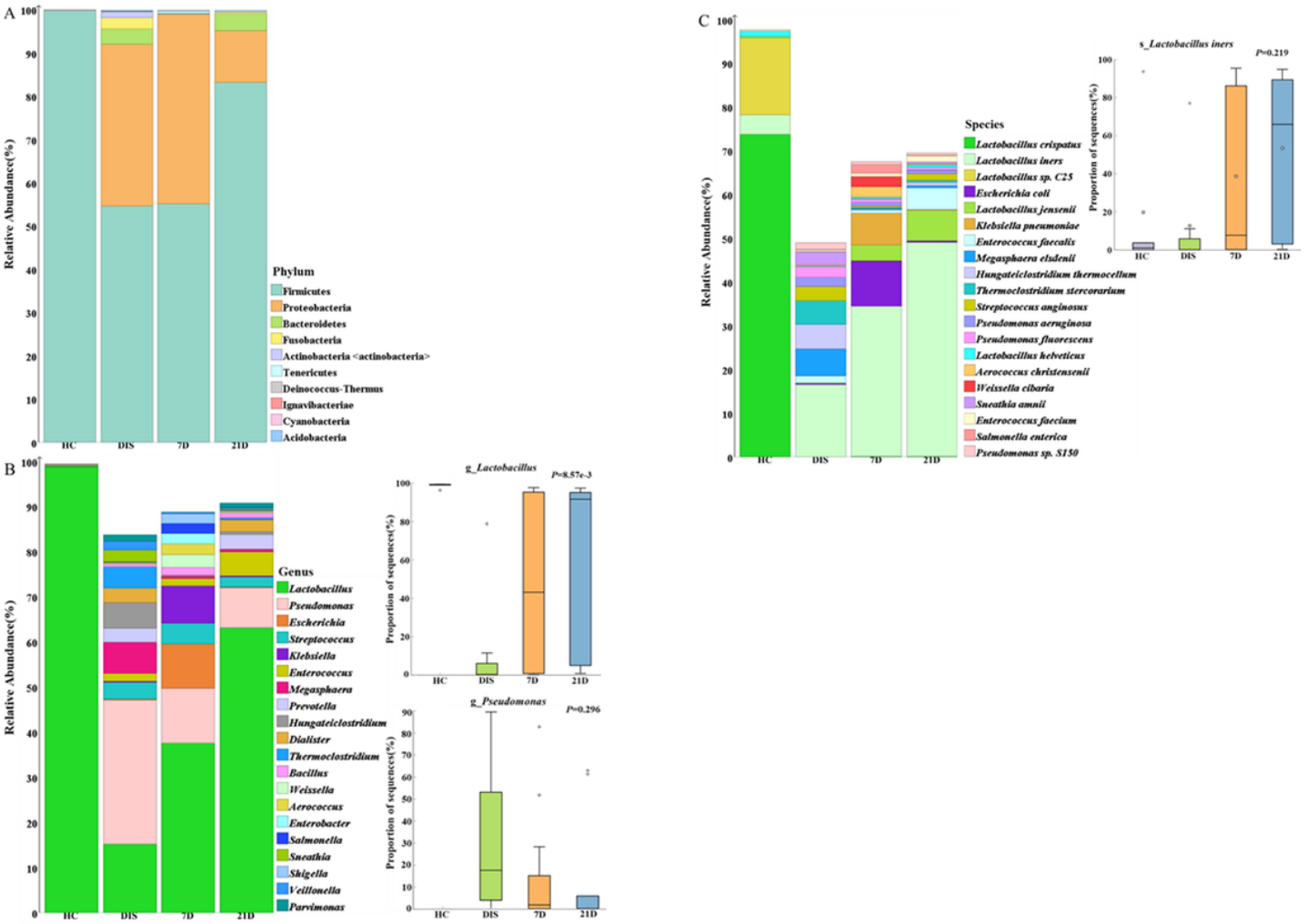

Figure 3

Composition of the vaginal microbiome in healthy control (HC) women and diseased patients (DIS) patients after treatment with gel for 0, 7, and 21 days.

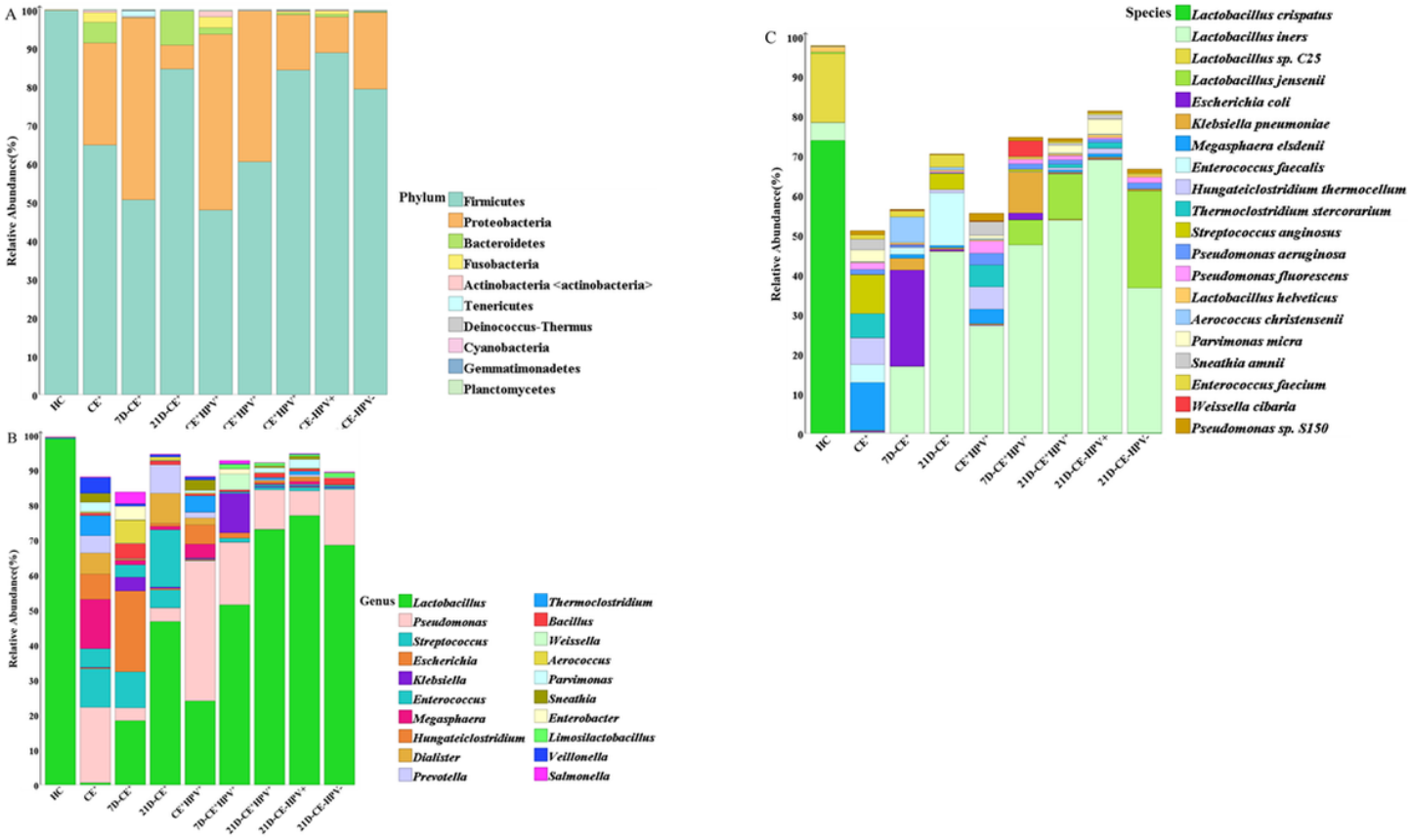

Figure 4

Species composition analysis for each group at the level of phylum, genus, and species (top 10 or 20). 21D-CE-HPV+group: patients who did not turn HPV− after 21 days of treatment with the gel in the $CE^{+}HPV^{+}$ group; 21D-CE-HPV−group: patients who turned HPV− after 21 days of treatment with the gel in the $CE^{+}HPV^{+}$ group.

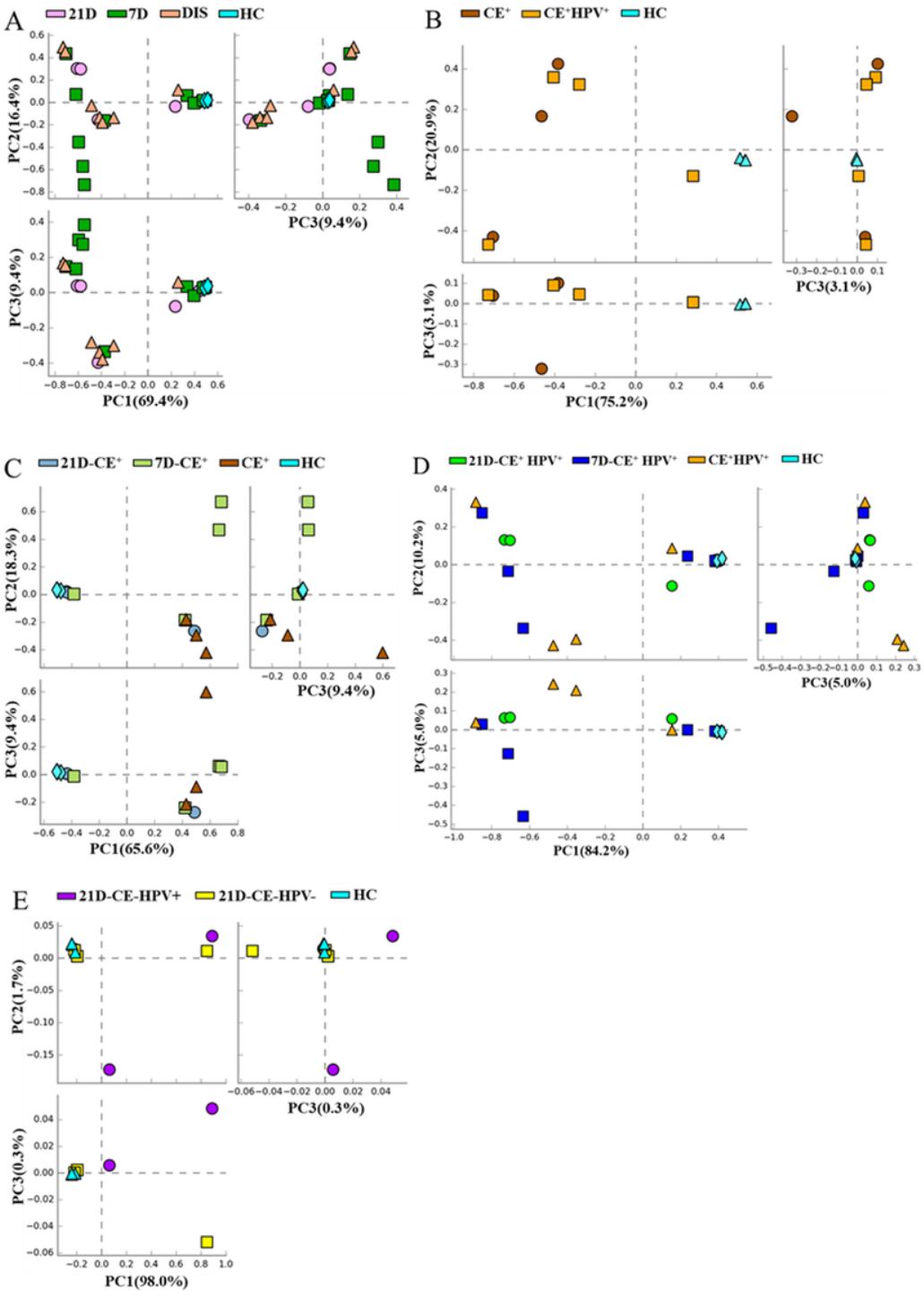

Figure 5

PCA analysis of each group at the genus level. The shapes of different colors represent the different sample groups, and the scale of the horizontal axis and the vertical axis is the relative distance, which has no practical significance. On the coordinate axis are eigenvalues that best reflect the variance value of this study. If the species composition of two groups of samples was more similar, the distance reflected in the PCA diagram would be closer.

Figure 6

Analysis of the significant difference of the vaginal microbiota at the species level ($p < 0.05$).

A: Significantly different microbiota between the HC and CE^+ groups; B: significantly different microbiota between the HC and CE^+HPV^+ groups; C: significantly different microbiota between the CE^+ and CE^+HPV^+ groups; D: significantly different microbiota between the CE^+ and 21D-CE^+ groups; E: significantly different

microbiota between the CE^+HPV^+ and 21D-CE^+HPV^+ groups; F: significantly different microbiota between the HC and 21D-CE^+ groups; G: significantly different microbiota between the HC and 21D-CE^+HPV^+ groups.

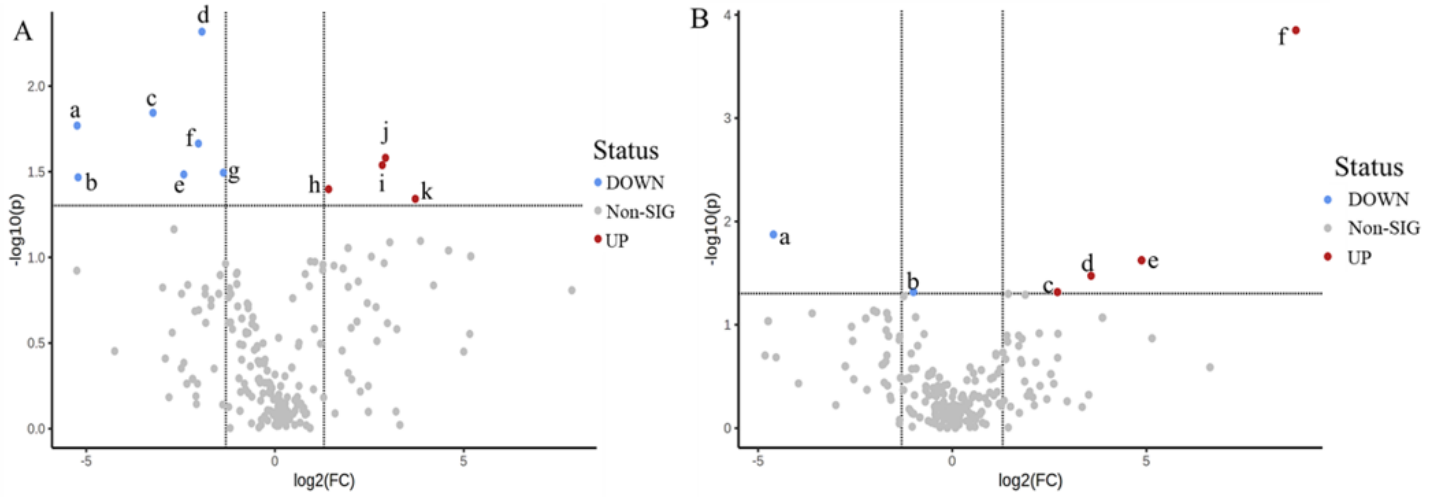

Figure 7

Univariate analysis of the vaginal metabolites in HC women and CE^+HPV^+ patients after treatment with gel for 0 and 21 days. A. Compared with the HC group, the red dots denote the metabolites that were significantly upregulated, while the blue dots denote the metabolites that were significantly downregulated, and the gray dots denote no significant difference in the CE^+HPV^+ group. a: Beta-nicotinamide adenine dinucleotide, b: adenosine, c: stachyose, d: cholic acid, e: urate, f: indolelactic acid, g: L-tryptophan, h: Dl-threitol, i: *m*-cresol, j: pipecolate, k: 2-hydroxy-2-methylbutyric acid. B. Compared with the CE^+HPV^+ group, the red dots denote the significantly upregulated metabolites, while the blue dots denote the significantly downregulated metabolites, and the gray dots denote no significant difference in the 21D-CE^+HPV^+ group. a: Histamine, b: acetoin, butanoate, isobutyrate, c: isovalerylcarnitine, d: beta-nicotinamide adenine dinucleotide, e: 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2-pyrocatechuic acid, 3,4-dihydroxybenzoate, gentisic acid, protocatechuic acid, f: epicatechin.

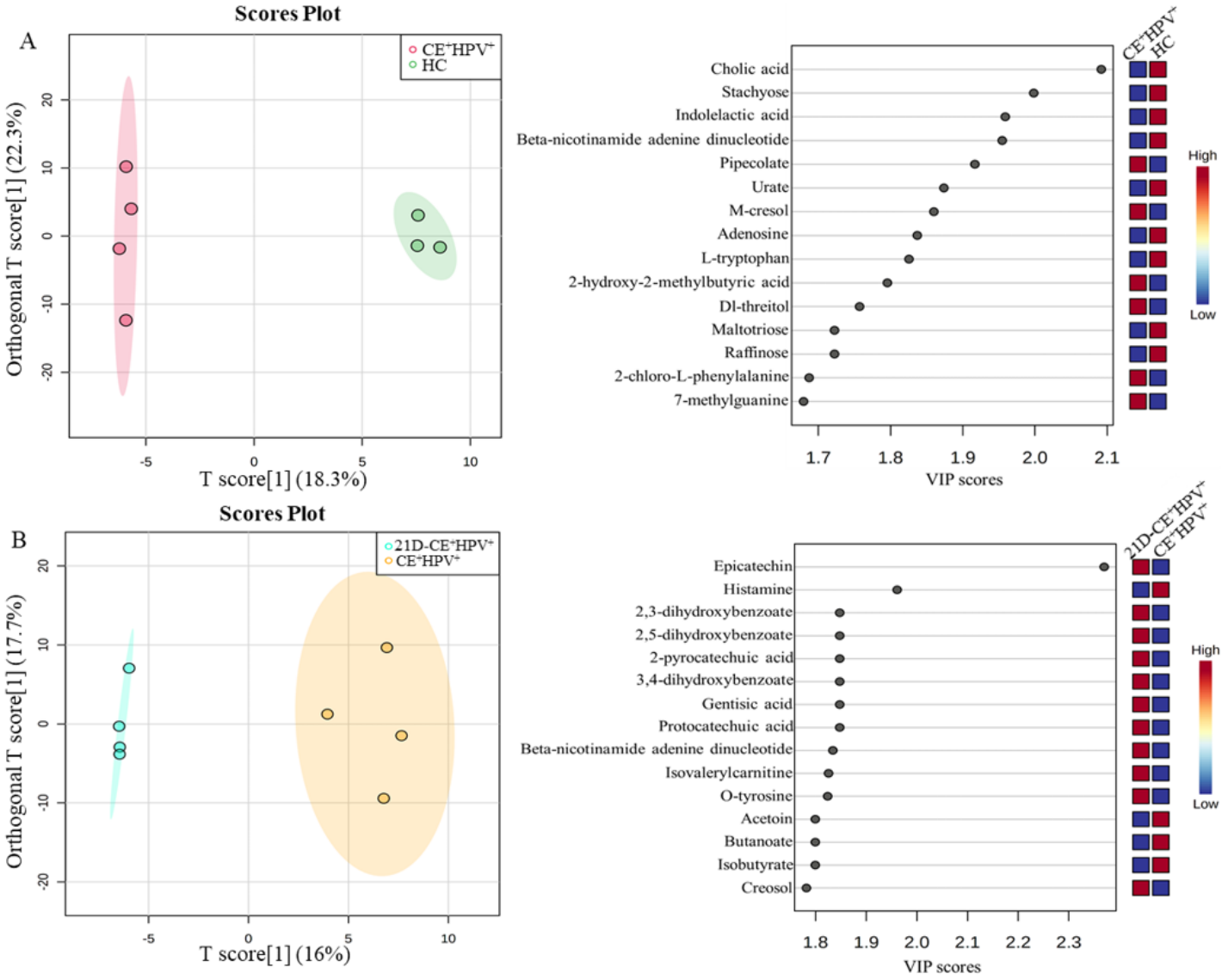

Figure 8

OPLS-DA analysis between the HC and CE$^+$HPV$^+$ groups and the CE$^+$HPV$^+$ and 21D-CE$^+$HPV$^+$ groups. The VIP score shows the influence intensity and explanatory ability of differences in metabolite accumulation on the classification and discrimination of each group of samples.

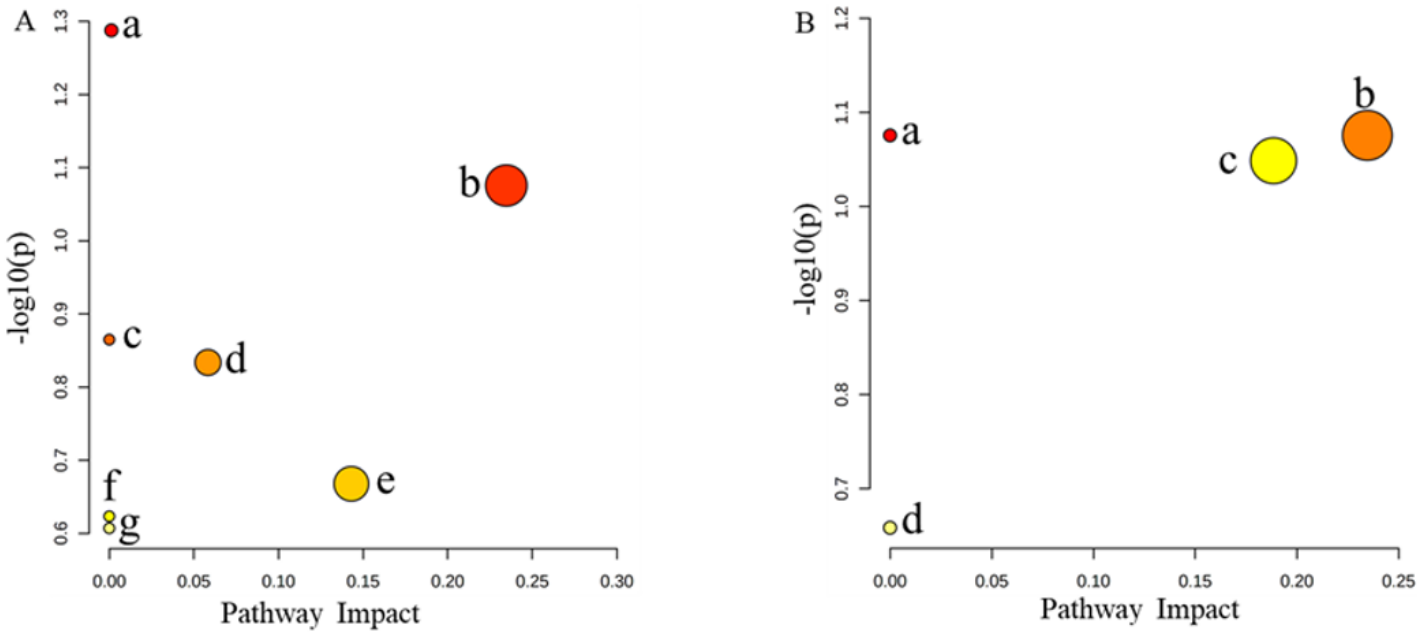

Figure 9

Summary of the metabolic pathway analysis associated with potential biomarkers.

A: Analysis of pathways associated with the metabolites with significant differences between the HC and $CE^{+}HPV^{+}$ groups. a: Purine metabolism, b: nicotinate and nicotinamide metabolism, c: lysine degradation, d: galactose metabolism, e: tryptophan metabolism, f: primary bile acid biosynthesis, g: aminoacyl-tRNA biosynthesis. B: Analysis of pathways associated with the metabolites with significant differences between the $CE^{+}HPV^{+}$ and 21D-$CE^{+}HPV^{+}$ groups. a: butanoate metabolism, b: nicotinate and nicotinamide metabolism, c: histidine metabolism, d: tyrosine metabolism.

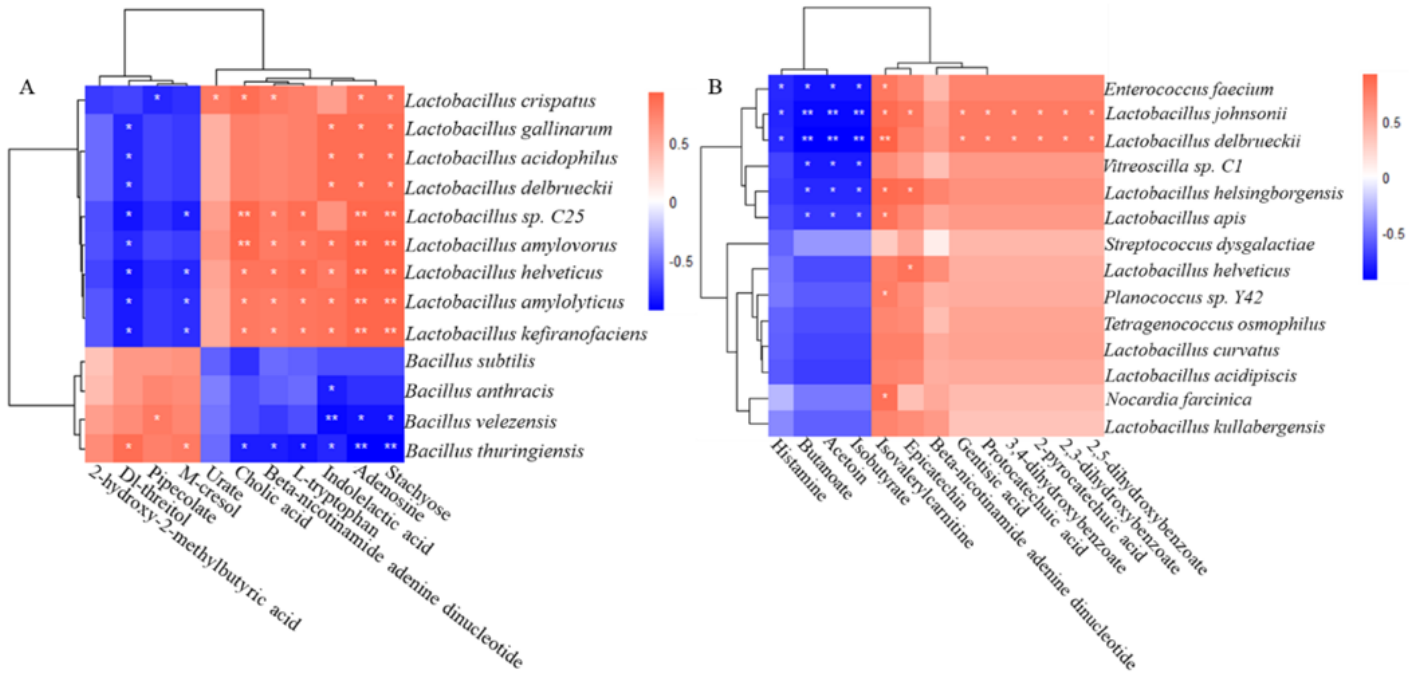

Figure 10

Spearman's correlation analysis of the differentially expressed microbiota and metabolites. A: Spearman's correlation analysis between the HC and $CE^{+}HPV^{+}$ groups. B: Spearman's correlation analysis between the $CE^{+}HPV^{+}$ and 21D-$CE^{+}HPV^{+}$ groups.

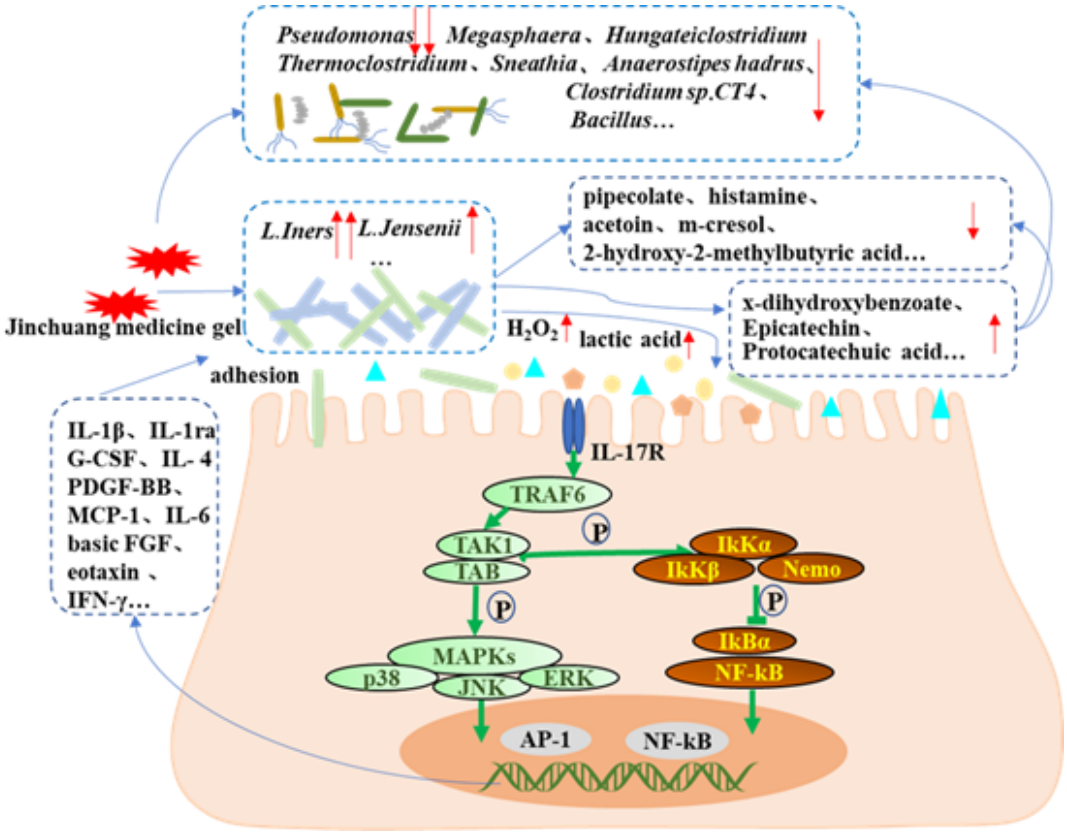

Figure 11

Prediction of the mechanism involved in patients who received treatment with Jinchuang medicine gel.