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RESEARCH ARTICLE

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Characterization of the vaginal microbiome during cytolytic vaginosis using high-throughput sequencing

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Abstract

Background: Cytolytic vaginosis (CV) is a common disease that results in pruritus, dyspareunia, and vulvar dysuria. However, the pathological mechanisms of the disease are still unclear. Compared to traditional methods, high-throughput sequencing can obtain more accurate qualitative and quantitative information on the microbiome.

Methods: We collected 75 samples from 32 healthy women (average age 44 ± 8) and 43 patients with CV (average age 38 ± 8). We used high-throughput sequencing of the 16S rRNA V3-V4 region to characterize and compare the vaginal microbiota of patients with CV and healthy women and to identify potential biomarkers for CV.

Results: The vaginal pH of patients with CV was ≤3.8, and the vaginal concentration of H_2O_2 was ≥2 μmol/L. Colony densities of *Lactobacillus* spp. in patients with CV ranged from +++ (5-30) to ++++ (>30) and were significantly higher than those in healthy women. High-throughput sequencing showed that *Lactobacillus* was the most prominent genus both in patients with CV and in healthy women, with abundances of 83.8% and 97.2%, respectively ($P < 0.001$). *Lactobacillus crispatus* was more abundant in patients with CV, whereas *Lactobacillus* sp. L-YJ was more abundant in healthy women, with area under the curve (AUC) values of 0.9375 and 0.8379, respectively.

Conclusion: The abundance of *Lactobacillus* spp. in CV patients was significantly different from that of healthy patients. Two suitable biomarkers, *L.* crispatus and *Lactobacillus* sp. L-YJ, were identified. These results will be useful for the identification of women at risk of serious illness before they develop obvious symptoms.

KEYWORDS
cytolytic vaginosis, high-throughput sequencing, vaginal microbiomes

1 | INTRODUCTION

Cytolytic vaginosis (CV) has received little attention because it is often confused with aerobic vaginitis (AV),[1-3] bacterial vaginosis (BV),[4-6] vulvovaginal candidiasis (VVC),[7-9] and trichomonas vaginitis (TV).[10-12] Clinical symptoms of CV include pruritus, dyspareunia, vulvar dysuria, and more pronounced cycling during the ovulatory and luteal phases.[13-16] Diagnostic criteria of CV include the absence of *Trichomonas* spp., *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera* spp., *Sneathia* spp., and *Prevotella* spp. and an increase in *Lactobacillus spp.* Under the influence of estrogen, glycogen is deposited in the vaginal epithelial cells as

Haihong Xu and Xueying Zhang contributed equally to this work.

TABLE 1 Characteristics of the study population

	CK	CV
Age (average, median)	44, 42	38, 37
16S sequences (average, median)	21 665, 20 650	21798, 1629
Observed_species (average ± SD)	57.31 ± 34.33	49.28 ± 26.71
Shannon (average ± SD)	1.82 ± 1.03	1.65 ± 0.5
Simpson (average ± SD)	0.5 ± 0.22	0.51 ± 0.13
Chao1 (average ± SD)	80.7 ± 37.51	79.3 ± 37.63
ACE (average ± SD)	83.6 ± 38.42	82.27 ± 30.69
Goods coverage (average ± SD)	0.998 ± 0.001	0.998 ± 0.001

Lactobacillus converts glucose to lactic acid.[17-20] Large numbers of *Lactobacillus* spp. also increase hydrogen peroxide (H_2O_2) and other antibacterial substances and decrease vaginal pH and bacterial diversity.[21-25]

High-throughput sequencing of the region of the bacterial 16S rRNA gene is a powerful tool for assessing and comparing the structure of microbial communities at a high phylogenetic resolution. 16S rRNA sequencing can obtain more accurate qualitative and quantitative information on the microbiome, and it is also Short-read and cost-effective, moreover, marker gene analysis is frequently used for broad studies that involve a large number of different samples.[26] The goal of this study was to use high-throughput sequencing to identify biomarkers for CV.

2 | MATERIAL AND METHODS

2.1 | Specimen collection

Vaginal secretion samples were obtained using aseptic cotton swabs from 43 patients with CV (CV group) and 32 healthy women (CK group). The samples were stored in phosphate-buffered saline (PBS) at 4°C and were sequenced within 48 hours. Patients were recruited at Women's Hospital School of Medicine Zhejiang University, and the healthy women were recruited at Sir Run Run Shaw Hospital School of Medicine, Zhejiang University, China. Patients were excluded if they used antibiotics. This study was approved by the Institutional Review Board of Sir Run Run Shaw Hospital School of Medicine, Zhejiang University, and Women's Hospital School of Medicine, Zhejiang University. All research was performed in accordance with all relevant guidelines and regulations.

2.2 | DNA extractions

DNA was extracted using the E.Z.N.A.® Stool DNA Kit (D4015, Omega Bio-Tek, Norcross, GA, USA) according to manufacturer's instructions. This kit was designed to recover trace amounts of DNA from samples and has been shown to be effective for the preparation of DNA from most bacteria. Nuclease-free water was used as the blank. Total DNA was eluted in 50 μL elution buffer and stored at −80°C until PCR assessment by LC-Bio Technology Co., Ltd (Hangzhou, China).

2.3 | PCR amplification and 16S rRNA sequencing

The V3-V4 region of the 16S rRNA gene was amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′).[27] The 5′ ends of the primers were tagged with sample-specific barcodes and universal sequencing primers. PCR amplification was performed in a 25 μL reaction mixture containing 25 ng of template DNA, 12.5 μL PCR premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. The PCR conditions for the amplification of the prokaryotic 16S fragment consisted of an initial denaturation at 98°C for 30 seconds; 35 cycles of denaturation at 98°C for 10 seconds, annealing at 54 or 52°C for 30 seconds, and extension at 72°C for 45 seconds; and a final extension at 72°C for 10 minutes. The PCR products were confirmed by electrophoresis on a 2% agarose gel. Throughout the DNA extraction process, ultrapure water was used in place of template DNA as a negative control to exclude false-positive PCR results. PCR products were purified using an AxyPrep PCR Cleanup Kit and quantified using Promega QuantiFluor. The pooled amplicons were prepared for sequencing, and the size and quantity of the amplicon library were assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. Libraries were sequenced on a 300PE MiSeq.

2.4 | Data analysis

Samples were sequenced by LC-Bio Technology Co., Ltd. Paired-end reads were assigned to samples based on their unique barcode, and samples were truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (v1.2.8).[28] Quality filtering on raw tags was performed using specific filtering conditions to obtain high-quality clean tags with FastQC. Verseach (v2.3.4)[29] was used to filter chimeric sequences and to assign samples with ≥97% sequence similarity to the same operational taxonomic units (OTUs). The representative sequence of each cluster is selected to represent the operational taxonomy units (OTUs).[30] Representative sequences were chosen for each OTU, and taxonomic data were assigned to each representative sequence using the Ribosomal Database Project (v11.5)[31-33]and NCBI classifier. OTU abundance data were normalized using a standard sequence number corresponding to the sample with the least number of

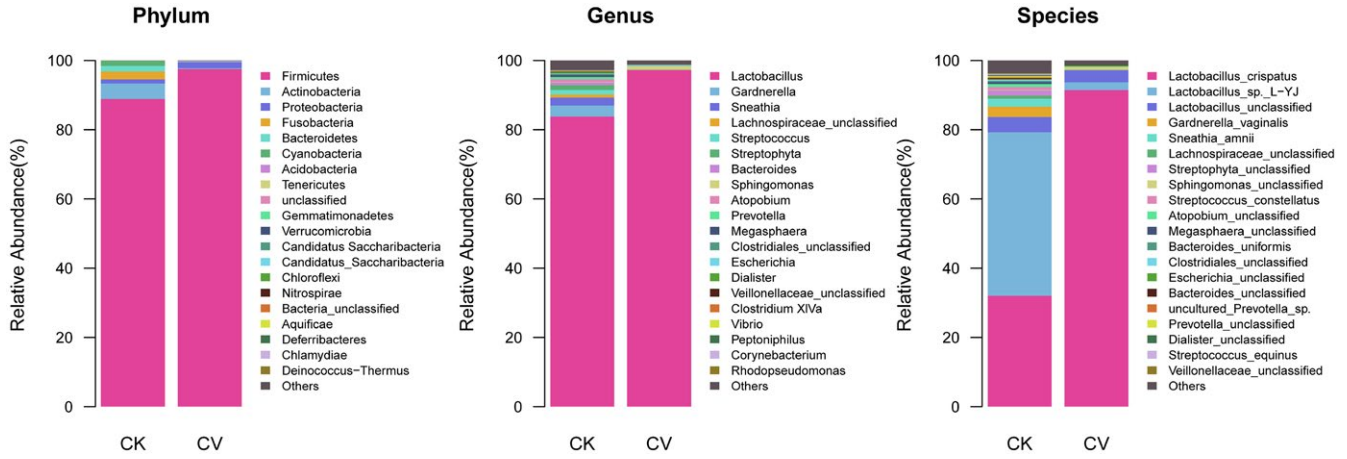

FIGURE 1 Composition of the vaginal microbiomes of healthy women (CK group) and cytolytic vaginosis (CV) patients (CV group). Relative abundance is shown at the phylum, genus, and species levels

sequences. These indices were calculated for our samples using QIIME (v1.8.0).[34] We used R packages to create receiver operating characteristic (ROC)[35,36] curves and other images and conducted functional prediction analyses using PICRUSt.[37] We used STAMP (v2.1.3)[38,39]for statistical analyses of differences.

3 | RESULTS

3.1 | OTU Sequence diversity and richness

Characteristics of women from groups CV and CK are shown in Table 1. We obtained 21 665 reads from women in the CK group and 21 798 reads from women in the CV group. Using a cutoff of 97% sequence similarity, we identified 645 OTUs. Because alpha diversity is often used to reflect the diversity of a particular environment or ecosystem based on the richness and uniformity of species and the depth of sequencing, we assessed alpha diversity using seven common indicators[40] (Table 1).

3.2 | Taxonomic variation at the phylum, genus, and species levels

Although the vaginal microbiota of all women in the study was characterized by high levels of *Firmicutes*, their abundance was significantly higher in the CV group (97.53%) than in the CK group (88.85%; $P = 0.0054$). In contrast, the abundance of *Actinobacteria* was 4.44% in the CK group and 0.27% in the CV group. Similarly, the abundance of *Fusobacteria* was 2.28% in the CK group and 0.01% in the CV group (Figure 1. Phylum). Diversity was also higher in the vaginal microbiomes of the CK group, which included *Gardnerella* (3.19%; $P = 0.008$), *Sneathia* (2.26%; $P = 0.0001$), and *Streptophyta* (1.44%; $P = 0.41$) (Fig 1. Genus).

Lactobacillus was the most prominent genus detected in both the CK (83.8%) and CV groups (97.2%; $P < 0.001$). In addition to the overall difference in the abundance of *Lactobacillus* between the CK and CV groups, differences were observed in the abundances of specific *Lactobacillus* species. In the CK group, 60% of *Lactobacillus* species in the vaginal microbiome were *Lactobacillus* sp. L-YJ and 40% were *L. crispatus*, whereas in the CV group, 97.5% of *Lactobacillus* species were *L. crispatus* and 2.5% were *Lactobacillus* sp. L-YJ.

Microbial diversity at the species level was also higher in the CK group (Fig 1. Species). Anahtar et al[41] demonstrated a strong link between high levels of diversity within cervicovaginal bacterial communities and genital inflammation in vivo in both cross-sectional and longitudinal studies of young South African women. Using high-resolution taxonomic identification, they reported several cervicotypes, one of which was characterized by *L. crispatus* and another by *Lactobacillus iners*. Virtanen et al sampled 10 Finnish women representing populations with diverse clinical characteristics. Six of these women had *Lactobacillus*-dominated microbiota, four had *L. iners*-dominated microbiota (community state type III), and two had *L. crispatus*-dominated bacteria (community state type I).

3.3 | Differential analysis of microbiota using linear discriminant analysis (LDA) effect size (LEfSe)

We investigated differences in the microbiomes of the two groups using linear discriminant analysis (LDA) effect size (LEfSe) with a *P* value cutoff of 0.05 for Kruskal-Wallis and pairwise Wilcoxon's tests and a threshold of 4.0 for the logarithmic LDA score. We found that the diversity of the CK group was approximately twice that of the CV group. Differences in the microbial communities of women in the CK and CV groups, including for *Lactobacillus* sp. L-YJ and *L. crispatus*, are shown in Fig 2.

3.4 | Suitable biomarkers for CV identified using ROC plots

ROC curves combining sensitivity and specificity with graphic methods and the area under the curve (AUC) were calculated to evaluate

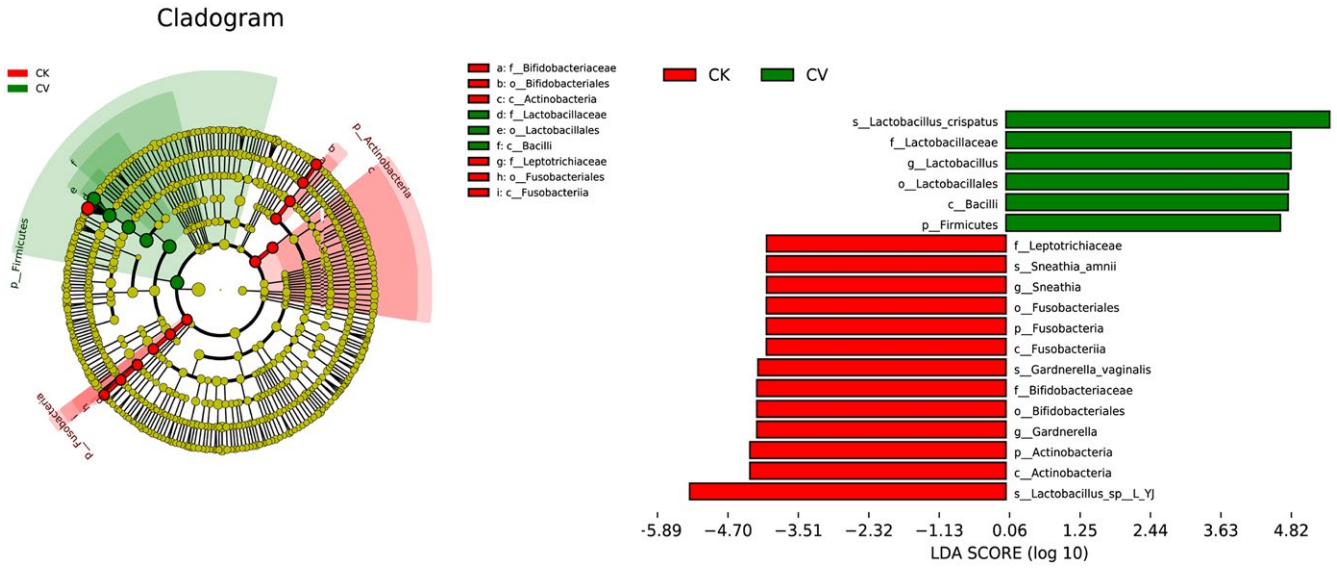

FIGURE 2 Cladogram and histogram of linear discriminant analysis (LDA) scores computed for differentially abundant genera between cytolytic vaginosis (CV) patients (CV group) and healthy women (CK group). Genera enriched in the CV group are indicated by a positive LDA score, whereas genera enriched in the CK group have a negative score ($P < 0.05$)

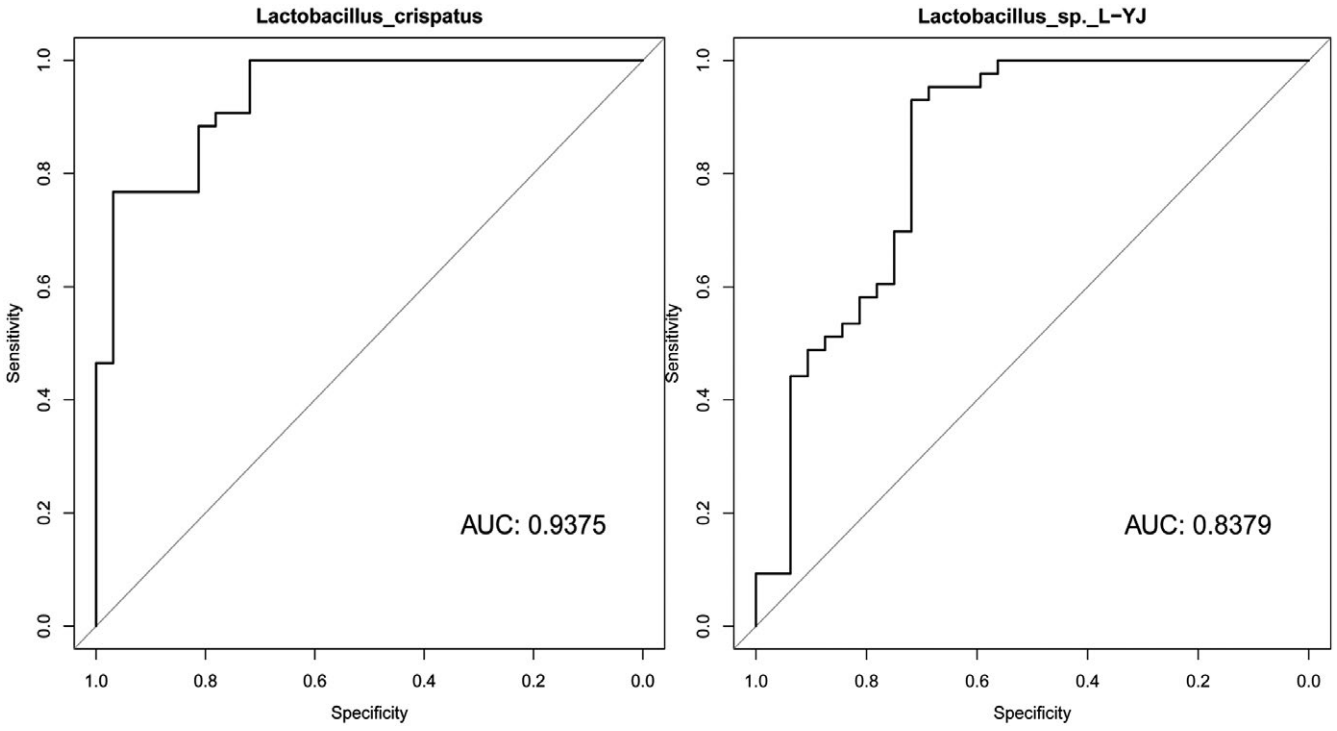

FIGURE 3 The abundance of two microbial biomarkers (*Lactobacilli crispatus* and *Lactobacillus* sp. L-YJ) differed significantly between cytolytic vaginosis (CV) patients (CV group) and healthy women (CK group). The area under the curve (AUC) values from receiver operating characteristic (ROC) curves were 0.9375 for *L. crispatus* and 0.8379 for *Lactobacillus* sp. L-YJ, respectively

the accuracy of diagnoses. Results were considered accurate for AUC values between 0.7 and 0.9 and highly accurate for AUC values >0.9. Using these criteria, we determined that *L. crispatus* and *Lactobacillus* sp. L-YJ could be used as biomarkers to distinguish between the CK and CV groups (Fig 3). Using ROC curve analysis, Ki Ho Hong et al[42] assessed the microbiomes of women in South Korea and reported that *Lactobacillus* spp. had an AUC value of 0.8559, which was slightly higher than our results for *Lactobacillus* sp. L-YJ (0.8379) but lower than our results for *L. crispatus* (0.9375). The reason for the differences between their results and our findings could

be related to differences in the vaginal microflora between women from China and South Korea.

3.5 | PICRUSt analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology identified six level 2 and 3 KEGG categories, including metabolism of cofactors and vitamins, nucleotide metabolism, carbohydrate metabolism, translation, replication and repair, and membrane transport. The most enriched KEGG categories in the CV group were carbohydrate metabolism, which included seven level 3 pathways, and membrane transport, which included two level 3 pathways. In particular, citrate (TCA) cycle, pyruvate metabolism, starch and sucrose metabolism, glycolysis/gluconeogenesis, and phosphotransferase system (PTS) pathways were significantly enriched in the CV group ($P < 0.01$; Fig 4). These results were supported by PICRUSt analyses and by P values for Welch's t-tests computed using STAMP.

4 | DISCUSSIONS

This study aimed at examining the variability between the microbiomes of patients with CV and healthy women to accurately detect CV and avoid inappropriate treatment and provided a comprehensive overview of the vaginal microbiome of patients with CV and healthy women in China using short-read, high-throughput sequencing of the V3-V4 region of the bacterial 16S rRNA gene. In our study, we found that the vaginal microbiomes of patients with CV were significantly less diverse than those of healthy women. *Lactobacillus* spp. were highly abundant in all women but were more abundant in CV patients than in healthy women ($P < 0.05$). These results support previous reports that CV is characterized by the abundant growth of *Lactobacillus* spp., which leads to lysis of vaginal epithelial cells at the molecular level. We also identified two useful biomarkers, *L. crispatus* and *Lactobacillus* sp. L-YJ, to identify CV. Our results indicated that *Lactobacillus* sp. L-YJ was the predominant *Lactobacillus* species in the vagina of healthy women. However, *L. crispatus* was the major *Lactobacillus* species in patients with CV. *Lactobacillus* sp. L-YJ

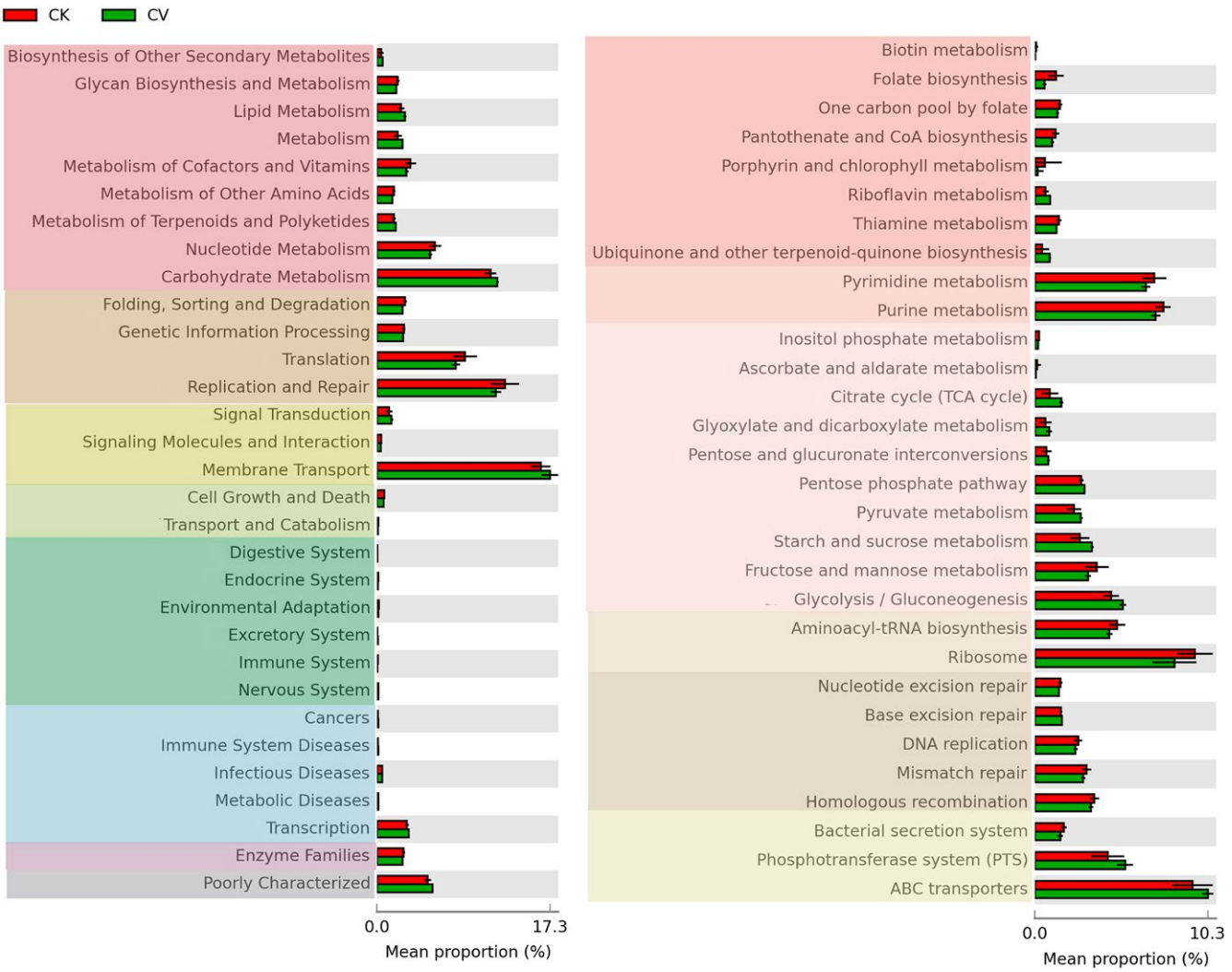

FIGURE 4 Analysis of differences in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways between cytolytic vaginosis (CV) patients (CV group) and healthy women (CK group) using PICRUSt. KEGG level 2 is shown on the left, and KEGG level 3 is shown on the right

is a necessary element of the normal vaginal environment of healthy women and may control the overgrowth of other organisms. The overgrowth of organisms can lead to vaginal epithelial tissue damage. Indeed, the abundant growth of *L. crispatus* appears to be a key factor in the development of CV.

Balanced microbiota in healthy women protects not only against ascending infections or HIV acquisition, but also against prematurity.[43-45] There are more than 200 bacterial species in the normal and the abnormal vaginal microbiota, and the vaginal microbiota is influenced by genes, ethnic background, and environmental and behavioral factors. Only several species of *lactobacilli* are dominate in healthy vagina. They support a defense system together with antibacterial substances, cytokines, defensins and others against dysbiosis, infections and care for a normal pregnancy without preterm birth. The disturbed vaginal microbiota may cause various vaginal diseases such as bacterial vaginosis (BV), aerobic vaginitis (AV), and CV. BV, which cause by the strains include *Atopobium vaginae*, *Clostridiales* and *Gardnerella vaginalis* develop in different mixtures and mumbers polymicrobial biofilms on the vaginal epithelium, while AV is dominated by aerobic bacteria such as *Streptococcus agalactiae* and *Escherichia coli*.[46]

The vaginal microbiome must be kept in a complicated balance. Internal and external factors that affect this balance and change the composition of the vaginal microbiome may lead to the development of CV. Therefore, assessing the microbiome may help to reduce the incidence of CV.

ETHICAL APPROVAL

This study was approved by the Institutional Review Board of Sir Run Run Shaw Hospital School of Medicine, Zhejiang University, and Women's Hospital School of Medicine, Zhejiang University. All research was performed in accordance with all relevant guidelines and regulations.

INFORMED CONSENT

The human materials used were vaginal swab samples, and all patients provided written informed consent.

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