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Longitudinal analysis on the ecological dynamics of the cervicovaginal microbiome in hrHPV infection

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ABSTRACT

The cervicovaginal microbiome (CVM) is a dynamic continuous microenvironment that can be clustered in microbial community state types (CSTs) and is associated with women's cervical health. *Lactobacillus*-depleted communities particularly associate with an increased susceptibility for persistence of high-risk human papillomavirus (hrHPV) infections and progression of disease, but the long-term ecological dynamics of CSTs after hrHPV infection diagnosis remain poorly understood. To determine such dynamics, we examined the CVM of our longitudinal cohort of 141 women diagnosed with hrHPV infection at baseline with collected cervical smears at two timepoints six-months apart. Here we describe that the long-term microbiome dissimilarity has a positive correlation with microbial diversity at both visits and that women with high abundance and dominance for *Lactobacillus iners* at baseline exhibit more similar microbiome composition at second visit than women with *Lactobacillus*-depleted communities at baseline. We further show that the species *Lactobacillus acidophilus* and *Megasphaera genomosp type 1* associate with CST changes between both visits. Lastly, we also observe that *Gardnerella vaginalis* is associated with the stability of *Lactobacillus*-depleted communities while *L. iners* is associated with the instability of *Megasphaera genomosp type 1*-dominated communities. Our data suggest dynamic patterns of cervicovaginal CSTs during hrHPV infection, which could be potentially used to develop microbiome-based therapies against infection progression towards disease.

1. Introduction

Microbiomes interact with their hosts and form symbiotic ecosystems that are associated with the host's health and with disease [1,2]. In women, the cervicovaginal microbiome (CVM) has been classified into microbial communities or community state types (CSTs) according to their microbial composition [3–5]. In healthy women, the CVM consists predominantly of *Lactobacillus* species that protect the cervical epithelium from microbial pathogens such as *Gardnerella vaginalis*, high-risk human papillomavirus (hrHPV), and HIV [3, 4, 6]. *Lactobacillus*-dominated (LDO) CSTs such as I, II, and V, dominated by *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*, respectively, have been associated with protection against cervicovaginal diseases. In contrast, CST III (dominated by *Lactobacillus iners*) and *Lactobacillus*-depleted (LDE) CST IV have been associated with a higher susceptibility to infections and hrHPV-induced neoplasia [7–10]. These associations have been mostly described in cross-sectional studies, and longitudinal analyses are still needed to fully assess the relationship between these communities and cervical health.

CSTs are dynamic and transitions between them occur regularly depending on cervical hygiene and the microenvironment conditions [3]. It was previously established that LDO microbiomes are more stable than LDE microbiomes in women who tested negative for hrHPV [11–13]. The dynamics of CSTs during hrHPV infection, however,

Abbreviations: BV, bacterial vaginosis; CiRNAseq, circular probes-based RNA sequencing; CSTs, community state types; CVM, cervicovaginal microbiome; hrHPV, high-risk human papillomavirus; JSD, Jensen-Shannon distance; LAC-CSTs, *Lactobacillus acidophilus*-containing CSTs; LDO, *Lactobacillus*-dominated; LDE, *Lactobacillus*-depleted; NILM, negative for intraepithelial lesions or malignancy; URC, unique read counts.

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remain poorly described. Research into the weekly dynamics of the CVM in hrHPV-positive women has revealed that CST IV exhibits high transition rates to other CSTs [14]. Nevertheless, in most women, hrHPV infections are cleared by the host immune system and assessing short-term CST dynamics might not provide sufficient information on the association between CSTs and hrHPV infection. In a longitudinal study, our group recently evaluated the association of the CVM with hrHPV infection progression in a six-month period and observed that the CVM composition correlated with the infection outcomes [15]. Yet, we did not explore the long-term dynamics of CSTs during infection independently of progression and neither investigated the species associated with the stability of CSTs. A detailed assessment into this high-resolution microbiome data might provide valuable insights into the CVM and hrHPV infections.

Microbiomes represent promising targets for the development of therapies against human diseases [16,17]. Fecal microbiota transplants have been successfully applied in the treatment of recurrent *Clostridium difficile* infections in the gut [18,19], demonstrating that microbiome-based therapies can be useful against dysbiosis and infections. Recent studies are focusing on the development of probiotics and vaginal microbiome transplants to treat bacterial vaginosis (BV), hrHPV infections, and cervical disease [3, 20, 21]. In terms of hrHPV infections, the use of probiotics aims to restore *Lactobacillus* dominance in the microbiome (CSTs I, II, or V) to promote viral clearance and disease regression [20]. The efficacy of those treatments will rely on the adequate selection of bacterial species for probiotics, long-lasting clinical response, and the ecological dynamics of the microbiome [22–24]. Thus, there is an unmet need to identify microbial species that can be potentially used to create an anti-hrHPV environment and restore a healthy CVM.

Subgroups of CSTs I, III, and IV have been recently identified and their composition suggests potential dynamics between communities and the continuity of the CVM [3, 5, 25]. CSTs I and III are classified into subgroups A and B, with both B subgroups characterized by colonization of *Lactobacillus acidophilus* and high abundance of *L. jensenii* relative to the A subgroups [25]. CST I-B has been associated with hrHPV negative conditions, while CST III-B is observed in hrHPV infections and cervical neoplastic lesions [25,26]. These *Lactobacillus acidophilus*-containing CSTs (LAC-CSTs, I-B and III-B) have been suggested to be transitional CSTs between LDO microbiomes (I, II, III, V) and between LDO (I, III) and LDE (IV) microbiomes, however, longitudinal studies on the dynamics of these CST subgroups have not been performed to date [25]. Furthermore, CST IV has been classified into subgroups A, B, and C [4, 5]. CST IV subgroups A and C are dominated by a wide range of species, which suggests a dynamic ecosystem [3,25]. CST IV subgroup B is dominated by *Megasphaera genomosp type 1* and has been associated with hrHPV-induced cervical intraepithelial neoplasia (CIN2 +) [25,26]. Interestingly, hrHPV infections have been associated with microbiome shifts and the occurrence of CST IV, which may indicate certain CST dynamics occurring during infection [27]. Amplicon-based sequencing technologies struggle with the identification of these microbial species and communities due to the high level of sequence identity of the small subunit rRNA of these relevant species with other similar species in the CVM [28,29]. Nonetheless, sequencing techniques such as shotgun metagenomics and circularizing probes-based RNA sequencing (ciRNAseq) can provide in-depth insights into the CVM composition and can be applied to study the dynamics of CSTs in health and disease and identify potential microbiome-based therapies against hrHPV-induced carcinogenesis [26, 30, 31].

In this study, we investigate the temporal stability of CSTs in women with a hrHPV-positive diagnosis at baseline and an initially negative cytology in the Dutch population-based cervical cancer screening program and define their long-term dynamics over a six-months period. We describe the dynamics of the CVM and define distinctive associations with the temporal changes of the microbiome.

2. Material and methods

2.1. Study subjects and inclusion criteria

A total of 141 women participating in the Dutch population-based cervical cancer screening program and diagnosed with hrHPV infection were enrolled in the study [15]. Women participating in the screening program were informed that residual material could be used for anonymous research and had the opportunity to opt out. Only residual material from women who did not opt-out was included. At first visit (V1, time = 0 months), 141 cervical smears in PreservCyt were collected, processed, and sequenced for microbiome profiling [26]. At second visit (V2, time = 6 months), all 141 women returned for sample collection, and their cervical smears in PreservCyt were also processed for microbiome profiling [26]. Five milliliters of each cervical cell suspension were centrifuged for 5 min at 2500g, and the pellet dissolved in 1 ml of Trizol reagent (Thermo Scientific). RNA was isolated through standard procedures and dissolved in 20 μl nuclease-free water. We routinely processed a maximum of 2 μg of RNA for DNase treatment and cDNA generation, using SuperscriptII (Thermo).

2.2. HrHPV identification and genotyping

HrHPV testing was performed with the Roche Cobas 4800 test, according to the manufacturer's recommendations in the Department of Medical Microbiology at Radboudumc [32].

2.3. CiRNAseq microbiome profiling and output analyses

High-resolution microbiome profiling was performed on ~ 50 ng of cDNA/DNA using the ciRNAseq technology [25, 26, 33]. Single-molecule molecular inversion probes (smMIPs) designed to bind to VRs in the 16 S and 18 S rRNA genes of microbial species in the CVM were mixed with cDNA in a capture hybridization reaction and were circularized via a combined primer extension and ligation reaction. Circularized probes were subjected to PCR with barcoded Illumina primers. After purification correct-size amplicons, quality control, and quantification [34], a 4 nM library was sequenced on the Illumina Nextseq500 platform (Illumina, San Diego, CA) at the Radboudumc sequencing facility. Reads were mapped against reference regions of interest in our Cervicovaginal Microbiome Panel containing 341 microbial species using the SeqNext module of JSI Sequence Pilot version 4.2.2 build 502 (JSI Medical Systems, Ettenheim, Germany). The settings for read processing were a minimum of 50% matching bases, a maximum of 15% mismatches, and a minimum of 50% consecutive bases without a mismatch between them; for read assigning, the threshold was a minimum of 95% of identical bases with the ROIs. All identical PCR products were reduced to one consensus read (unique read counts, URC) using a unique molecular identifier. We set an arbitrary threshold of at least 1000 URC from all smMIPs combined in an individual sample, below which we considered an output non-interpretable. For microbial annotation, species with two reactive smMIPs were annotated when 100% of the specific set of smMIPs had URC. Species with three or more reactive smMIPs were annotated when more than 50% of their specific set of smMIPs had URC [26].

2.4. Microbiome assessment and analyses

CSTs designation was performed through unsupervised cluster analyses using ClustVis [25, 35, 36]. CSTs were classified into five major groups (I to V) and the subgroups of CSTs I, III, and IV based on microbiome composition [25].

SankeyMATIC software was used to visualize the temporal microbiome analysis. The microbiome variation in the six-months period within a woman was obtained through a Jensen-Shannon Distance (JSD) calculation in the philentropy R package [37]. JSD values give a

measure of similarity between samples (i.e., by calculating the distance between samples) from the same woman. Lower JSD values indicate more similar microbial communities and conversely, high values indicate a less similar community. Correlations with JSD values were calculated with the lares R package [38]. The Bray-Curtis distance between microbiomes was calculated with the vegan R package [39]. Network analyses were performed using the CARlasso R package [40]. CARlasso implements the chain graph model [41] and allows to infer a complex network structure that represents both interactions among microbial taxa and the effects of a set of covariates. CARlasso estimates a network that represents the conditional dependence structure of a multivariate response (e.g., microbial abundances) while simultaneously estimating the conditional effect of a set of covariates that correlate with the network (e.g., microbiome similarity, diversity, and instability).

2.5. Statistical analysis

GraphPad Prism v9.5.1 (GraphPad Software, Inc., USA) was used to analyze datasets and determine Odd ratios and the Shannon's diversity indices. The statistical significance of differences between groups and paired differences between visits were calculated using the Kruskal-Wallis or Mixed-model effect tests, respectively, followed by a Benjamini-Hochberg test correction for multiple comparisons. Paired data was analyzed by fitting a mixed model as implemented in GraphPad Prism v9.5.1. Repeated measures ANOVA cannot handle missing values. This mixed model uses a compound symmetry covariance matrix and is fitted using Restricted Maximum Likelihood (REML). In the absence of missing values, this method gives the same p values and multiple comparisons tests as repeated measures ANOVA. In the presence of missing values (missing completely at random), the results can be interpreted like repeated measures ANOVA.

Correlations between bacterial abundances and JSD as well as the Odd ratios were followed by a Benjamini-Hochberg test correction for multiple comparisons using the MultipleTesting Tool [42]. A Mann-Whitney U test was performed for unpaired analyses between two groups.

3. Results

3.1. Long-term dynamics of the cervicovaginal microbiome in hrHPV infection

In the Netherlands, screened women with a positive hrHPV result and negative cytology, are invited for a repeat cytology test after 6 months. We first characterized the CVM composition through unsupervised cluster analyses in all samples collected at first (V1) and second (V2) visits ($n = 141$) [15] and determined the dynamics of microbial communities between the two collection timepoints. The distribution of the samples over the CSTs at both visits is visualized in a Sankey diagram in Fig. 1a. Microbiomes with CSTs I, III, and IV mostly maintained the same community over the two timepoints with on average 70% staying in the same CST and no significant differences between CSTs (Fig. 1a).

The Jensen-Shannon distances (JSD) between the microbiome composition per baseline CSTs at both visits are shown in Fig. 1b. The lower the JSD, the higher the similarity in microbial composition between both timepoints. Of note, baseline CSTs may not be the same at V2 (Fig. 1a). We found that CSTs I ($q < 0.0001$, Kruskal-Wallis test) and III ($q < 0.0001$) had a significantly higher microbiome similarity than CST IV over a six-months period (Fig. 1b). When considering the CST subgroups, we also noticed the same observations shown in Fig. 1b in these CSTs (Supplementary Figure 1a). We also calculated the Shannon's diversity indices for all CVM at both visits and found a significant positive correlation with the JSD values in both directions, i.e. from V1 to V2 and from V2 to V1 [Spearman $r = 0.35$, $p < 0.0001$ (V1), Spearman $r = 0.27$, $p = 0.001$ (V2)] (Fig. 1c and Supplementary Figure 1b), suggesting that high diversity correlated with less similar microbiomes over a six-months period, and there is no trend towards increasing or decreasing diversity.

Next, we correlated the microbial abundances of the most prevalent species in the CVM at V1 with the JSD to identify the species associated with the similarity of the microbiomes. We noticed that *Atopobium vaginae*, *G. vaginalis*, *Dialister micraerophilus,* ($q < 0.001$) and other CST IV-associated bacteria ($q < 0.20$) positively correlated with the JSD values, while *L. iners* ($q < 0.001$) and *L. jensenii* ($q < 0.20$) exhibited a

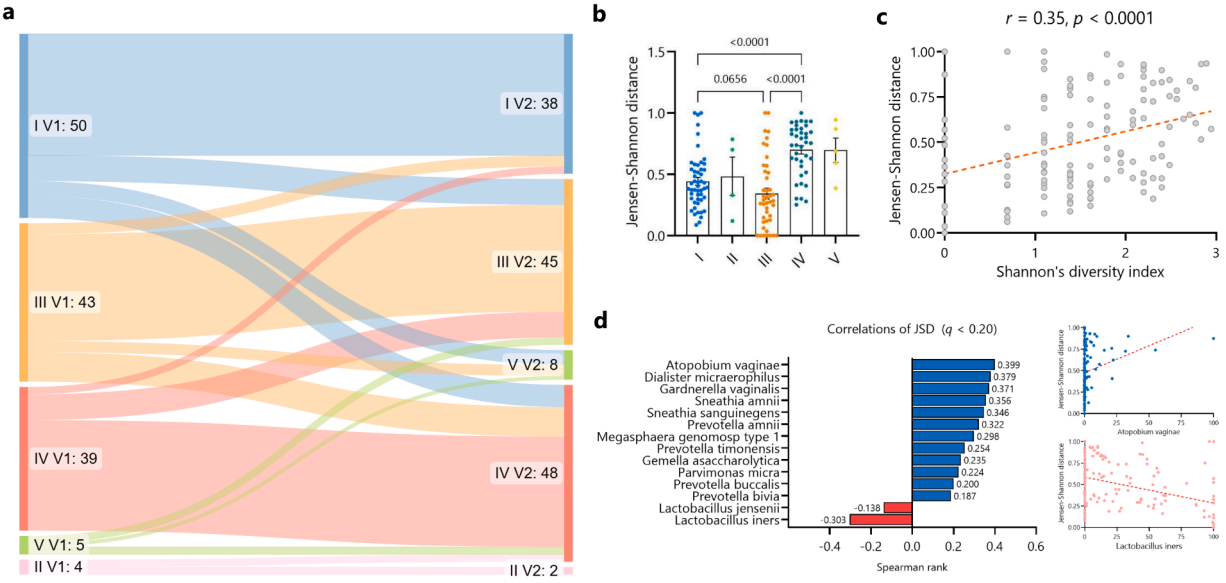

Fig. 1. Long-term dynamics of the cervicovaginal microbiome during hrHPV infection. a. The microbial dynamics of CSTs (I, II, III, IV, and V) between both visits (V1, V2) and communities are displayed using a Sankey diagram. b. Analysis of the similarity of the microbiome composition between both visits through the Jensen-Shannon distance (JSD) between baseline CSTs. c. Spearman correlation analysis between the Shannon's diversity indices of all CVM at V1 and the JSD between V1 and V2. d. Spearman correlation analysis of the relative abundances for the top 14 species in the CVM at V1 with the most significant correlations with the JSD values ($q < 0.20$, after Benjamini-Hochberg correction for multiple comparisons) and the correlation analyses between the JSD and *L. iners* or *A. vaginae* abundances. Differences in JSD (b) were analyzed with a Kruskal-Wallis test followed by the Benjamini-Hochberg test correction. q values are shown in b.

negative correlation (Fig. 1d). These analyses show that the species composition associates with the microbiome similarity in a six-months period, which corroborates the associations observed at the community-level.

3.2. The species *Lactobacillus acidophilus* and *Megasphaera genomosp type 1* associate with microbial community shifts in hrHPV infection

Since we observed that CSTs transitioned between each other in a six-months period (Fig. 1a), we evaluated the odds for the CST composition at baseline of shifting to a different microbial community at second visit (Fig. 2a). For this analysis, baseline microbiomes that maintained the same CST group and subgroup at V2 were designated as stable ($n = 67$) and those that shifted to a different CST group and subgroup were defined as unstable ($n = 74$). OR analyses were calculated with a Fisher's exact test comparing all CSTs and associations with $q < 0.20$ were considered significant. This assessment revealed that women with *Lactobacillus acidophilus*-containing CSTs (LAC-CSTs, I-B and III-B) (OR 4.92, 95% CI 1.94–12.36, $q = 0.0022$, Fisher's exact test) or *Megasphaera genomosp type 1*-dominated CST (IV-B) (OR 2.89, 95% CI 1.00–7.60, $q = 0.1172$, Fisher's exact test) at baseline were significantly associated with a microbial community shift at V2 when compared to other CSTs at V1. Alternatively, women with a baseline *Lactobacillus iners*-dominated CST (III-A) were significantly associated with a stable community composition in a six-month period when compared to other CSTs at V1 (OR 0.19, 95% CI 0.07–0.50, $q = 0.0022$, Fisher's exact test) (Fig. 2a).

Next, we estimated the relative abundances of *L. acidophilus* and *M. genomosp type 1* within unstable (U) and stable (S) microbiomes to evaluate whether their abundances correlated with the stability of the CVM between both visits. We found that both *L. acidophilus* [$q = 0.0621$ (V1), $q = 0.1071$ (V2) Kruskal Wallis test] and *M. genomosp type 1* [$q = 0.0770$ (V1), $q = 0.1479$ (V2)] were significantly more abundant in unstable CVMs when compared to stable CVMs at V1 and V2 (Fig. 2b). The high abundance of these species at V1 in unstable microbiomes is consistent with the association of LAC and IV-B CSTs with community shifts shown in Fig. 2a. Likewise, the high abundance of these species at V2 (Fig. 2b) suggests that CVMs with low abundance of these species at V1 may also transition to LAC and IV-B CSTs at V2, reinforcing the hypothesis that these communities may represent transitional states during hrHPV infection [25]. We then performed a network analysis with the list of species that exhibited the highest correlations with the microbiome similarity index (Fig. 1d) and evaluated their relationship with the microbiome instability to identify additional species associated with microbiome shifts (Supplementary Figure 2). Here we confirmed the positive association of *L. acidophilus* and *M. genomosp type 1* with unstable communities (Fig. 2), and observed that the species *Prevotella amnii*, *G. vaginalis*, and *L. jensenii* also exhibited a positive but weaker association with unstable communities. Alternatively, the species *Sneathia amnii*, *D. micraerophilus*, and *L. crispatus* showed a small negative association with unstable CSTs, indicating that they are associated with stable communities (Supplementary Figure 2).

3.3. Assessing the stability of microbial communities in hrHPV infection

The stability results at the level of the CST subgroups suggest that part of the reason we see transitions is that some of the CVMs are already more similar to the CST they transition to than the ones who do not. To examine this further, we analyzed baseline CVMs according to their community composition between timepoints. A PCA plot showed distinctive clusters for stable *Lactobacillus*-depleted microbiomes (LDE>LDE), stable *Lactobacillus*-dominated microbiomes (LDO>LDO), and LDE microbiomes that transitioned to LDO at V2 (LDE>LDO) (Supplementary Figure 3a). Further analyses consistently determined that all LDE>LDO samples had a significantly similar microbiome composition to LDO at V1 than LDE>LDE CVMs ($p = 0.0035$, Mann-Whitney U test). For the reverse, LDO>LDE transitions are less clear, only five LDO>LDE samples exhibited a relatively similar composition at baseline to LDE (Supplementary Figure 3b).

Next, since the stability of microbial communities are associated with specific species (Fig. 2), we wondered whether we could identify the species that correlated with the stability of communities that are mainly associated with health and disease, such as CSTs I-A, III-A, IV-A, and IV-B [25,26], and therefore could be potentially used in therapeutical approaches. To this purpose, we first compared the relative abundances of bacterial species that are typical in unstable (U) and stable (S) CSTs I-A ($n = 32$), III-A ($n = 27$), IV-A ($n = 15$), and IV-B ($n = 19$) at both visits. We did not find significant species associations with the stability of CSTs I-A, III-A and IV-A at V1 (Fig. 3a and Supplementary Figure 4). However, we observed that unstable CSTs I-A and III-A were significantly associated with high abundance for *L. acidophilus*, *D. micraerophilus*, and *G. vaginalis* at V2 (Supplementary Figure4).

In terms of the disease-associated CST IV subgroups, stable CST IV-A were associated with a significant increase in abundance for *G. vaginalis* from V1 to V2 ($q = 0.0332$, Mixed-effect model). Alternatively, unstable CST IV-A exhibited an increase in *M. genomosp type 1* from V1 to V2 ($q < 0.0001$, Mixed-effect model) (Fig. 3a). In addition, unstable CST IV-A also showed low abundance for *D. micraerophilus* ($q = 0.0609$, Kruskal Wallis test) and high abundance for *M. genomosp type 1* ($q < 0.0001$, Kruskal Wallis test) when compared to stable CST IV-A at V2 (Fig. 3a). Unstable CST IV-B had an increase in abundance for *L. iners* from V1 to V2 ($q < 0.0001$, Mixed-effect model) that was significantly higher than stable CST IV-B at both V1 ($q = 0.0525$, Kruskal Wallis test) and V2 ($q =$

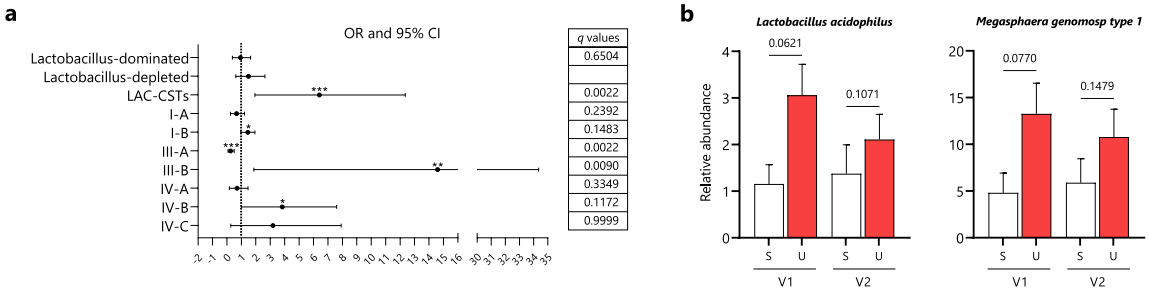

Fig. 2. Microbial communities and species associated with the stability of microbiomes in hrHPV infection. a. Odd ratios (OR) analyses of changes in microbial community subgroups between V1 to V2 reveals associations of *Lactobacillus acidophilus*-containing CSTs (LAC-CSTs, I-B, III-B) and CST III-A (*L. iners* dominance and higher abundance than in III-B) with unstable and stable communities, respectively. b. Analysis on the relative abundances of *L. acidophilus* and *M. genomosp type 1* in all CVM ($n = 141$) show that both species associate with unstable (U, red bars) microbiomes at V1 and V2. OR in a were analyzed through a Fisher's exact test followed by the Benjamini-Hochberg test correction for multiple comparisons. *, $q < 0.20$; **, $q < 0.01$; ***, $q < 0.003$. Differences in relative abundances between groups in b were analyzed through a Kruskal-Wallis test followed by a Benjamini-Hochberg test correction. q values are shown in a and b.

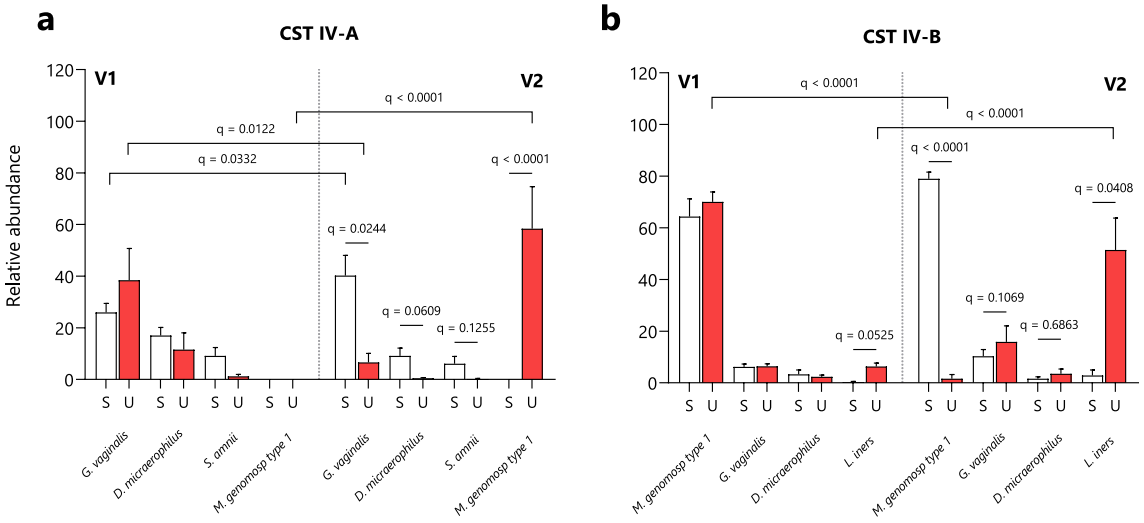

Fig. 3. Compositional stability of disease-associated CSTs during hrHPV infection. Analysis on the relative abundances of bacterial species in CSTs IV-A (a, $n = 15$) and IV-B (b, $n = 19$) show the species that associate with stable (S, white bars) or unstable (U, red bars) microbiomes at both visits (V1, V2). Error bars represent standard error of the mean ± s.e.m. Differences in relative abundances between groups were analyzed through a Kruskal-Wallis test followed by the Benjamini-Hochberg correction for multiple comparisons. Paired differences in relative abundances between visits were analyzed through a Mixed-effect model followed by the Benjamini-Hochberg correction. q values < 0.20 are considered significant.

0.0408, Kruskal Wallis test) (Fig. 3b). Overall, we determined that the species *G. vaginalis*, *M. genomosp type 1,* and *L. iners* were associated with the stability of CST IV subgroups in a six-months period.

4. Discussion

Based on high-resolution microbiome profiling of our longitudinal cohort of women with proven hrHPV infection at baseline, we here describe the ecological dynamics of CSTs and characterize their stability and change over a six-months period. In most hrHPV-positive women, CSTs can shift between each other, and represent transitional states in the continuous composition of the CVM [14,43]. To fully assess the relationship between the CVM composition with its long-term dynamics, we investigated these microbial associations at the species level. We found that *L. iners* was associated with a stable long-term microbiome composition at both community and species levels [14], while *L. acidophilus* was only associated with microbiome shifts at the community level. These differences may be explained because *L. iners* can dominate the CVM, reside in a highly diverse CST IV [4,25], and is associated with shifts between CST IV and LDO microbiomes as described in this study. Conversely, *L. acidophilus* has been mostly described as a co-resident species within CSTs I and III [25], which may result in shifts between LDO CSTs rather than to CST IV. Our data therefore corroborates proposed dynamics of cervicovaginal CSTs [25] and shows that *Lactobacillus acidophilus*-containing CSTs (LAC-CSTs, I-B and III-B) and *Megasphaera genomosp type 1*-dominated CST (IV-B) may represent transitional communities during hrHPV infection. *L. acidophilus* can colonize mucins-rich environments like CSTs I and III, which might provide better adhesion to vaginal epithelial cells and access to nutrients [24, 44–46]. This colonization, however, may result in a competition for nutrient sources like glycogen [24,47] and cause a decrease in abundance of the dominant bacterium [3,25]. *L. acidophilus* and CST I-B have been associated with hrHPV-negative conditions [26], however it is currently unknown whether LAC-CSTs represent transitional communities in uninfected conditions and additional longitudinal studies with hrHPV-negative women are needed.

Microbiomes with CST III-A (*L. iners* dominance) are relatively stable and do not seemingly shift to a different CST over a six-months period. Although LDO CSTs are generally associated with cervical health [48, 49], CST III has been the exception, and *L. iners* has been associated with hrHPV infections, viral-induced dysbiosis, and carcinogenesis [26, 50, 51]. Therefore, long-lasting CST III-A microbiomes possess a higher risk for disease outcomes than do CSTs I, II, and V [14, 25, 52]. *L. iners* can adapt to changes in the cervicovaginal ecosystem due to specific accessory genes that are not present in other *Lactobacillus* species, thereby allowing the bacterium to prevail in adverse conditions such as bacterial vaginosis (BV) [51, 53, 54]. CST III-A has a low abundance of *L. acidophilus*, *D. micraerophilus*, and *G. vaginalis* and colonization of these species in this community is associated with microbial shifts to LAC-CSTs or CST IV. The high stability of CST III-A in hrHPV-infected women described in this study shows that this community, its dominant species, and its co-resident species, might therefore represent a promising target for microbiome-based therapies against viral infection and cervical neoplasia [55,56]. Probiotics, phage therapy, or vaginal microbiome transplants could be used to promote a healthy shift from CST III-A to CSTs I, II, or V by increasing the abundance of *L. crispatus*, *L. acidophilus*, *L. gasseri*, or *L. jensenii* and preventing a detrimental shift to CST IV by inhibiting the growth of *D. micraerophilus* and *G. vaginalis* [3,57]. Furthermore, compared to LDO CSTs, LDE CSTs have a lower microbiome similarity over a six-months period, which was associated with its distinctive microbial diversity and *Lactobacillus* depletion [58]. CSTs IV-A and IV-B associate with hrHPV infections and CIN development, and their stability correlates with the abundance and diversity of co-resident bacterial species. This is important because a microbial community shift to healthy CSTs could also be stimulated by an initial treatment with *L. iners* followed by an increase of *L. crispatus*, *L. acidophilus*, *L. gasseri*, or *L. jensenii* abundances while inhibiting the growth of *G. vaginalis* and *D. micraerophilus*, which then may result in a protective cervicovaginal microenvironment against cervical disease [55, 59–61]. Additional studies are needed to test these hypotheses and dynamics *in vitro* and *in vivo*, and clinical trials will be essential to evaluate potential microbiome-based therapies against hrHPV-induced cervical disease.

The composition of the CVM can also transition frequently between communities [11], and previous longitudinal analyses have partly investigated the temporal dynamics of CSTs during hrHPV infection. In a study of hrHPV-positive women with vaginal swabs taking twice weekly for up to 4 months, Brotman et al. identified a high stability for CST III, which agrees with our findings over a six-months period [14]. However, we did not observe the same transition rates for CST IV, possibly due to

the longer study period in our analysis [14]. Recent research has determined that sampling over a long period can provide better insights into the cumulative long-term transition dynamics that shape the overall prevalence of CSTs within a population [56]. The dynamics of CSTs during hrHPV infection may also differ between short and long-term, which might be associated with early clearance of the virus. In former longitudinal analyses performed over a six-month period, hrHPV-positive women have been associated with stable CSTs III and IV, and hrHPV-negative women with stable LDO CSTs, particularly CST I [62,63]. Similarly, in this same longitudinal cohort, our group recently described that women with a non-progressive hrHPV infection were characterized by a LDO CVM, while women with a progressive hrHPV infection displayed a LDE CVM, particularly a CST IV-A [15]. Moreover, we also found that women with a stable CST I over a six-month period were associated with non-progression of hrHPV infections and women with a stable CST IV were associated with the development of cervical abnormalities. Interestingly, at the six-month follow-up visit, we observed that there were more hrHPV-negative women in the non-progression group than in the progression group, suggesting that LDO communities and stable CSTs I might be also associated with hrHPV clearance [15]. These dynamics, however, may differ for up to 2 years after infection diagnosis [43]. Mitra et al. observed that the CVM of hrHPV-positive women with either persistent lesions or regression was relatively stable irrespective of the infection outcome at 12 and 24 months follow-up visits [43], which may indicate that cervical lesions that persist beyond six-months might not dictate the composition and stability of the CVM, and other possible mechanisms such as the hrHPV-induced dysbiosis [27] and changes in bacterial transcriptional activities [24] should be considered and studied. Furthermore, even though there are diverse hrHPV genotypes prevalent worldwide, the association of each hrHPV genotype with the microbiome dynamics is not clear yet. HPV16 infections has been mostly associated with a CST IV composition when compared to other hrHPV genotypes [8,64], however, larger study cohorts and longitudinal analysis are needed to confirm these observations to fully elucidate the relationship between hrHPV and the microbiome dynamics during disease.

The strengths of our study are the use of the ciRNAseq technology for species-level microbiome profiling and the longitudinal design of hrHPV-positive women over a 6-months period [26]. Some potential limitations may include a relatively small cohort size and the absence of hrHPV-negative women to compare the microbial dynamics in health conditions. We were also unable to control for phase of the menstrual cycle or antibiotic use during the study, which impact on CST composition [65]. Lastly, we did not determine the pH and Nugent score of the cervical smears, which are known factors that correlate with CSTs [12,66].

5. Conclusions

In summary, we have studied the long-term ecological dynamics of the classical CSTs and the recently defined subgroups of CSTs I, III, and IV in hrHPV-positive women. More studies into the long-term relationship of the microbiome and hrHPV infections and the development of disease are required to direct future therapeutic approaches. The temporal CST dynamics described in this study further support the continuum concept of the CVM [67,68], which emphasizes on the co-occurrence of species in the CVM and the continuous dynamic interactions and composition of microbial communities. Thus, our data promotes the use of high-resolution microbiome profiling in the study of the CVM in health and disease, which has been useful in the identification of bacterial species relevant in the cervicovaginal microenvironment and the continuity of the CVM.

Ethics statement

The Central Committee on Research Involving Human Subjects (CCMO) and the National Institute for Public Health and Environment (RIVM) reviewed and granted approval before the start of the study (No. 2014–1295). All methods were performed in accordance with the Radboudumc ethical guidelines for using human samples, including the Declaration of Helsinki.

CRediT authorship contribution statement

Mariano A. Molina: Conceptualization; Data curation, Formal analysis, Investigation, Resources, Software, Visualization, Roles/Writing – original draft, Writing – review & editing. **Willem J. G. Melchers**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Karolina M. Andralojc**: Conceptualization, Supervision, Writing – review & editing. **William P. J. Leenders**: Conceptualization, Methodology, Resources, Software, Supervision, Writing – review & editing. **Martijn A. Huynen**: Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing non-financial interests but the following competing financial interests: William P. J. Leenders is CSO and shareholder of Predica Diagnostics.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.09.011.

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