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RESEARCH ARTICLE

Impact of the Probiotic on the Modulation of Vaginal Bacterial and Fungal Microbiota in HPV-Positive Women

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ABSTRACT

This study examined the effects of administering the probiotic *Lactiplantibacillus plantarum* Probio87 on the vaginal microbiota of HPV-positive women, highlighting alterations in bacterial and fungal communities and their potential influence on vaginal and overall health. In a double-blind, randomized, placebo-controlled study, participants consumed daily doses of Probio87 (1×10^9 CFU/sachet) or a placebo for 12 weeks. Vaginal swabs were collected, and DNA was extracted for 16S rRNA and the internal transcribed spacer (ITS) region amplification via PCR, followed by sequencing of bacterial and fungal microbiota. The probiotic group showed improved alpha and beta diversity, with an increase in beneficial *Lactobacillus* ($p = 0.014$) and *Phascolarctobacterium faecium* ($p = 0.041$), while decreasing the abundance of pathogens like *Streptococcus* and *Candida* ($p = 0.011$). Additionally, vaginal health questionnaires linked probiotic use to improved vaginal and mental health in HPV-positive women. *L. plantarum* Probio87 demonstrated clinical and psychological benefits in HPV-positive women by balancing the vaginal microbiome, reducing pathogenic species, and promoting the growth of beneficial microorganisms.

1 | Introduction

Human papillomavirus (HPV) is a double-stranded DNA virus. While most HPV infections resolve on their own, persistent infections cause 95% of cervical cancers, making it the fourth most common cancer in women. In 2022, an estimated 660 000 women were diagnosed, and 350 000 died globally [1]. In Malaysia, approximately 1740 women are diagnosed, and 991 die from the disease annually [2].

Trial Registration: ClinicalTrials.gov identifier: NCT05316064

The gut-vagina axis links gut microbiota (GM) with vaginal microbiota (VM), allowing GM to affect vaginal health by serving as a pathogen reservoir, modulating systemic inflammation, and altering hormone levels like estrogen [3]. A balanced VM supports vaginal health and reduces sexually transmitted infections, including bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), HPV, and human immunodeficiency virus (HIV) [4]. An in vitro model showed that bacteria inhibit *Candida albicans* biofilm formation, a key virulence factor, by suppressing fungal growth and hypha-associated genes [5]. VM also influences HPV infection and cervical cancer progression, with increased VM diversity and lower *Lactobacillus* abundance linked to HPV persistence [6]. Despite advances in understanding vaginal bacterial communities, knowledge of the fungal microbiome remains limited [7].

Probiotics—live microorganisms that benefit health—can alter the VM through the gut-vagina axis [8]. Probiotics enhance the epithelial barrier, boost mucin production, support immune development, and inhibit pathogen adhesion [9]. *Lactobacillus* species produce hydrogen peroxide and bacteriocins, which suppress pathogenic bacteria and fungi, thus helping prevent vaginal infections by restoring microbiome balance [10, 11]. Certain probiotics (*L. plantarum* and *L. fermentum*) showed potential for preventing or treating BV and VVC by inhibiting pathogens and reducing inflammation [12].

In the vagina, microbes exist in a finely tuned mutualistic relationship with the host and provide the first line of defense against the colonization by opportunistic pathogens. Throughout a woman's lifespan, the vaginal microbiota undergoes major changes associated with transitional reproductive periods such as puberty and menopause. Healthy vaginal microbiota is dominated by *Lactobacillus* spp., but a diverse array of other bacteria can be present in much lower numbers. Over 20 species of *Lactobacillus* have been detected in the vagina; however, in the majority of women, the healthy vaginal microflora contains one or two *Lactobacillus* species from a range of three or four species, mainly *L. crispatus, L. jensenii, and L. gasseri*. A diverse array of other bacteria, such as *Staphylococcus, Ureaplasma, Corynebacterium, Streptococcus, Peptostreptococcus, Gardnerella, Prevotella, Clostridium, Bacteroides, Mycoplasma, Enterococcus, Escherichia, Veillonella, Bifidobacterium, and Candida* [13], can be present but in much lower numbers. Women with *Lactobacillus*-poor flora have an increased susceptibility to sexually transmitted pathogens. Cross-sectional and longitudinal studies have demonstrated the association of vaginal microbiota with the prevalence and incidence of many viral sexually transmitted infections, such as HIV, HPV, herpes simplex virus, and cytomegalovirus infection [14]. Genital HPVs are the etiological cause in the development of cervical cancer. Studies involving BV and HPV found that women with BV and HPV had significantly developed cervical intraepithelial neoplasia. Conversely, a normal vaginal flora, dominated by *Lactobacillus* species, was strongly associated with a negative HPV test ($p < 0.001$) [15].

The presence of *Lactobacilli* in the vagina, which maintain an acidic environment (pH 3.8–4.5), also often inhibits the overgrowth of opportunistic yeast species. While *C. albicans* is the most commonly identified yeast in the vagina, other species such as *Candida glabrata*, *Candida tropicalis*, and *Candida parapsilosis* are also prevalent. These yeasts typically exist as commensals at low levels, contributing to the overall microbial diversity without causing disease [16]. However, their presence and abundance can be significantly altered in the context of certain diseases, such as HPV infection. Since HPV induces local immunosuppression and disrupts the vaginal microbiota, studies have demonstrated that HPV infection is associated with a marked increase in yeast colonization, particularly *Candida* spp., due to the suppression of local immune responses and changes in the vaginal environment that favor fungal overgrowth [17]. For instance, HPV infection has been shown to elevate vaginal pH, reducing the competitiveness of lactobacilli and creating a more favorable niche for yeast proliferation. This shift in microbial composition can lead to dysbiosis, characterized by decreased lactobacilli abundance and increased yeast dominance [18]. Moreover, specific yeast species such as *C. glabrata* and *C. tropicalis* have been reported to exhibit enhanced virulence in the presence of HPV. These species can form biofilms and produce proteases that degrade host mucosal barriers, exacerbating inflammation and potentially contributing to recurrent VVC. The interplay between HPV and yeast species may also involve synergistic interactions, where viral factors enhance the pathogenicity of fungi, leading to more severe clinical outcomes. For example, HPV-induced immunosuppression can impair the host's ability to mount an effective antifungal response, allowing yeast species to proliferate unchecked [17].

Understanding the complex interactions between bacterial and fungus species and HPV in the vaginal microbiota is essential for elucidating the mechanisms underlying dysbiosis. This knowledge informs the development of targeted interventions aimed at restoring microbial homeostasis and mitigating the risk of complications associated with both HPV and microbial overgrowth and provides a deeper understanding about specific molecular pathways involved in these interactions. In this study, we aimed to evaluate the potentials of probiotic in modulating vaginal microbiota of HPV-positive women, in hope that probiotic can be used as a natural strategy against vaginal dysbiosis.

2 | Experimental Section

2.1 | Study Design

This double-blind, randomized, placebo-controlled study assigned participants 1:1 to probiotic or placebo groups using a computer-generated list. Randomization was conducted by a blinded statistician, with the allocation sequence concealed from other staff, like the research team, until study completion. The process steps were outlined in Figure 1, following the approach described by Bespalov et al. [19]. The study adhered to the Declaration of Helsinki and received approval from the Medical Research and Ethics Committee, Ministry of Health Malaysia (Approval No. NMRR-21-1819-61300).

Probiotic (*Lactiplantibacillus* [previously *Lactobacillus*] *plantarum* Probio87) contained probiotic (10%) and carrier (90%), while placebo contained only carrier (non-GMO corn starch). Sachets of products containing the probiotic and placebo appeared as light-yellow powder. Both probiotic and placebo products are kept at a storage temperature range below 30°C

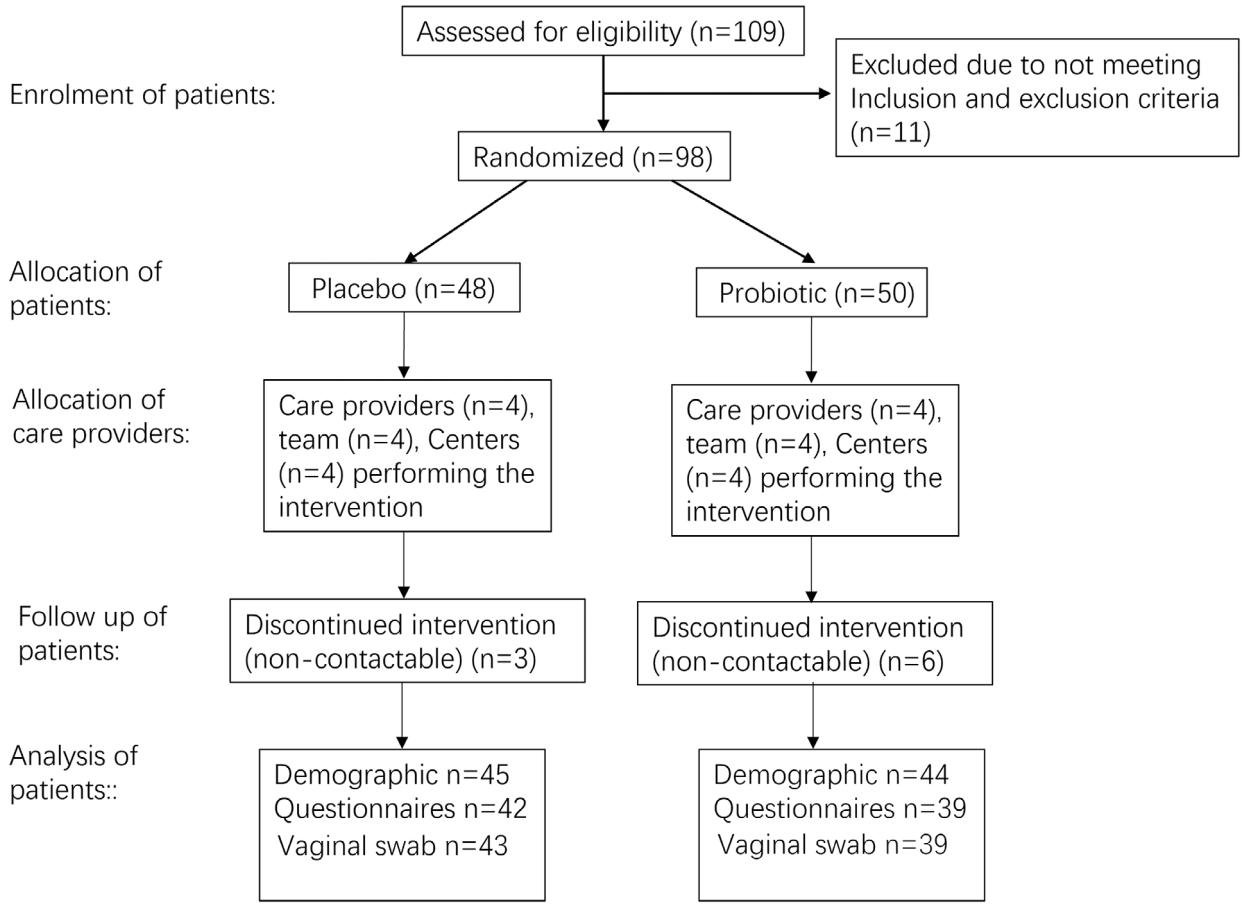

FIGURE 1 | The CONSORT flowchart details patient recruitment, randomization, and allocation.

and away from direct sunlight, according to the condition recommended by the manufacturer. The intervention consisted of orally administering one sachet/day of probiotic (9 log CFU/sachet) or placebo for 12 weeks.

2.2 | Participants

Before the trial, all participants provided written informed consent. Subjects were recruited in Malaysia from Hospital Seberang Jaya (Penang), Hospital Raja Perempuan Zainab II (Kelantan), Universiti Malaysia Sarawak (Sarawak), and through community outreach from March 2022 to March 2024. Eligible participants were sexually active women over the age of 26, as the Advisory Committee on Immunization Practices recommended HPV vaccination for females up to age 26 in 2006 [20]. Additionally, participants were confirmed HPV-positive for the L1 variant through PCR-based HPV genotyping via hospital screening and committed to completing the study. Exclusion criteria included long-term medication (6+ months), pregnancy, prior HPV vaccination, cervical intraepithelial neoplasia, or history of uterus/cervix removal.

2.3 | Sample Collection

Vaginal swab samples were collected at week 0 and week 12 via self-swabs. Each swab had a mark to indicate the depth of insertion into the vagina, to ensure consistency between all subjects. Upon insertion, swabs were rotated 10 times inside the vagina. Swabs were collected into a swab collection tube containing RNAlater solution (Qiagen, Hilden, Germany) for microbiota analysis. All tubes were capped tightly and stored at −80°C until further analyses.

Based on power analysis, the sample size was calculated for a parallel group study with one prevention and one placebo arm. At the start of this study, only three relevant studies examined the impact of probiotics on HPV. Based on subject counts from Ou et al. [21] and Palma et al. [22], each with 120 participants, and Verhoeven et al. [23], with 102 participants (twice the size of their pilot study), an average sample size of 114 participants was calculated, corresponding to 57 participants per group.

2.4 | Microbiota Analyses of Vaginal Swab Samples

2.4.1 | DNA Extraction

DNA from vaginal swab samples was extracted and purified by the TIANamp Genomic DNA Kit (DP304; TIANGEN Biotech, Beijing, China). Briefly, 500 µL of sample was centrifuged at 12 000 rpm for 3 min, resuspended in 200 µL Buffer GA and 20 µL Proteinase K, and then incubated at 56°C for 60 min. After adding 200 µL Buffer GB and incubating at 70°C for 10 min, 200 µL

ethanol (96%–100%) was added. The mixture was loaded onto a spin column, centrifuged, washed with 500 µL Buffer GD and twice with 600 µL Buffer PW, and then dried. DNA was eluted with 50 µL of DEPC-treated water (Bio Basic, Canada) after 10 min at 25°C. To prevent contamination, a sterile, DNase/RNase-free environment was maintained, including negative controls and laminar flow hood use. DNA quality was verified via NanoDrop 2000 (Thermo Scientific, USA).

2.4.2 | Microbiota Analyses

The V3–V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 341F (5'-CCTAYGGGRBGCASCAG-3') - 806R (5'-GGACTACHVGGGTWTCTAAT-3') [24], while the internal transcribed spacer (ITS) region-1 of the nuclear ribosomal coding cistron, part of the region formally proposed as a universal DNA barcode for fungi, was amplified with primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') [25] by thermocycler PCR system (GeneAmp 9700, ABI, San Diego, CA, USA). The PCR reactions were performed in triplicate with 20 µL mixture containing 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA, in the following sequence: 3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C, and a final extension at 72°C for 10 min. The PCR products were then extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor ST (Promega, Madison, WI, USA) according to the manufacturer's protocol. The purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

The 16S rRNA gene sequences were processed using QIIME v.1.9.1 (ref QIIME allows analysis of high-throughput community sequencing data) and USEARCH v.10.0 (ref Search and clustering orders of magnitude faster than BLAST). Raw FASTQ files were quality filtered by Trimmomatic and merged by USEARCH with the following criteria: removing of barcodes and primers, filtering of low-quality reads, and finding non-redundancy reads. The merged raw reads were at least 50 000 per sample. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE. The taxonomy for each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva 132 16S rRNA database using an 80% confidence threshold. Microbiota data were analyzed using Microbiome Analyst phyloseq-R package version 3.6.1 (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml) for different taxonomic levels. Mycobiome sequencing followed the protocol described by Li et al. [26]. Raw FASTQ ITS1 sequencing data were filtered to retain high-quality reads, with adapter sequences removed using cutadapt v1.4.1, and reads lacking the proximal primer sequence were excluded. Sequence reads were further processed by quality trimming, where any reads with an average quality score below 20 across a 3-base-pair sliding window were truncated and reads shorter than 100 bp were discarded. The remaining high-quality reads were aligned to the targeted host fungi (THF) ITS1 database using BLAST v2.2.22, and OTUs were identified at ≥ 97% identity using the pick_otus.py pipeline within the QIIME v1.6 framework. The alignment results were then summarized across all reads, with the accession identifiers of the ITS reference sequences used as surrogate OTUs and tabulated using a Perl script.

2.5 | Questionnaire Analysis

Eligible subjects meeting inclusion and exclusion criteria completed two Malay questionnaires: (i) a validated demographic questionnaire (baseline, week 0) [27] and (ii) vaginal health and sexual function questionnaires (weeks 0, 12). The latter included: (i) the Vaginal Assessment Scale (VAS) and Vulvar Assessment Scale (VuAS), with four items on vaginal and vulvar symptoms scored 0–3 (0 = none, 3 = severe), where lower scores indicate better health, validated for healthy and female cancer populations [28]; and (ii) the General Vaginal Health Questionnaire, adapted from the Vulvovaginal Symptom Questionnaire (VSQ), assessing 21 items on vulvovaginal, emotional, social, and sexual impacts [29]. The Malay version modified "vulvar" to "private parts" for cultural sensitivity.

2.6 | Statistical Analysis

Data were analyzed in SPSS version 24.0 (SPSS Inc., Chicago, IL). Due to the skewed, non-parametric data, the Mann–Whitney U test was used for scale data comparisons, and Spearman's rank correlation with rho (r) was used for non-parametric correlations. VAS, VuAS, and VSQ questionnaire correlation raw data are from our previous research paper, which was accepted by the journal Beneficial Microbes (Manuscript ID: BM-1188R1). All tests were two-sided, with $p < 0.05$ considered significant, and data are presented as mean ± standard error unless otherwise specified.

3 | Results and Discussion

3.1 | Baseline

A total of 109 subjects were screened for eligibility, with only 98 fulfilling the inclusion and exclusion criteria. Of the 98 subjects that were recruited, 9 subjects dropped out due to being uncontactable during the 12-week period, while 7 subjects were unable to provide complete sets of vaginal swab samples (due to menstruation thus unable to collect vaginal swab), yielding 82 subjects for final analyses of vaginal samples (Figure 1). Insignificant differences were observed in all the demographic characteristics of probiotic and placebo subjects ($p > 0.05$; Table S1). No adverse effects were reported throughout the study and no patients dropped out due to any complications.

3.2 | Bacteriome Sequencing

3.2.1 | Alpha Diversity

Alpha diversity measures differences within samples via the use of several indices. The Shannon and Simpson indexes measure

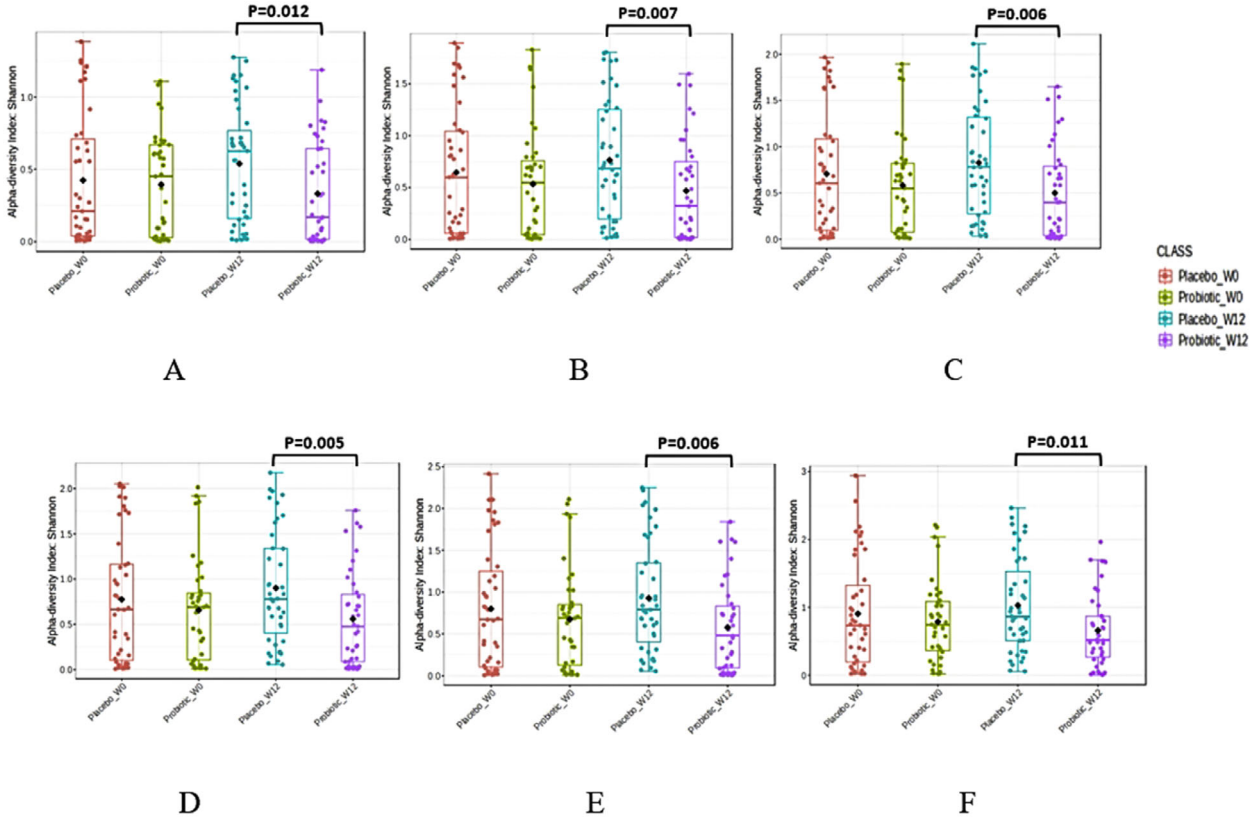

FIGURE 2 | Alpha diversity plots of vaginal bacteria at baseline (week 0) and week 12 after administration of probiotic *L. plantarum* Probio87 or placebo. Shannon index differences are shown for (A) phylum (W0, $p = 0.912$); (B) class (W0, $p = 0.448$); (C) order (W0, $p = 0.448$); (D) family (W0, $p = 0.542$); (E) genus (W0, $p = 0.592$); and (F) species (W0, $p = 0.89$). Boxes represent the median and interquartile range, with whiskers indicating range within that interval; outliers and individual sample values appear as dots. Mann–Whitney *U* test was used for analysis. $n = 82$ (Probio87 $n = 39$, placebo $n = 43$).

both richness and evenness, taking into account the frequency of each OTU. Higher Shannon or Simpson index values indicate higher diversity as influenced by both the number of OTUs and their relative abundances. At baseline, both groups showed similar Shannon indexes at levels of phylum ($p = 0.912$), class ($p = 0.448$), order ($p = 0.448$), family ($p = 0.542$), genus ($p = 0.592$) and species ($p = 0.89$) (Figure 2). However, at week 12, the placebo group showed higher Shannon indexes as compared to the probiotic group at all taxonomic levels studied ($p < 0.05$). A similar trend was observed for Simpson indexes across all taxonomic levels studied (Figure S1).

Our present data showed that the administration of Probio87 prevented the increase of within-group ecological diversity across different taxa and relative abundances, which was evidently increased in the placebo group after 12 weeks. This also indicated the presence of certain dominating OTUs which are described in more details via subsequent analyses below. Meanwhile, increasing diversity among the vaginal microbiota is generally indicative of an imbalanced microbiota which is associated with diseases and infections [30]. The composition of the vaginal microbiota plays a critical role in maintaining the acidic pH and preventing the overgrowth of harmful microorganisms. Women with BV have more diverse and heterogeneous communities, with higher levels of species richness and diversity of bacteria than women without BV [31].

3.2.2 | Beta Diversity

Beta diversity measures differences between samples. The Bray–Curtis Index considers both the co-occurrence and differential abundance of OTUs. Principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS), as measured by PERMANOVA for different taxonomic levels of family, genus, and species, showed insignificant changes at baseline between groups (Figure 3). However, differences were observed at week 12 between probiotic and placebo groups for PCoA at these taxonomic levels (family $p = 0.045$, genus $p = 0.037$, species marginally $p = 0.062$). Similarly, despite the insignificant differences between groups at week 0, differences were observed at week 12 between the probiotic and placebo groups for NMDS at levels of family ($p = 0.043$) and genus ($p = 0.036$), while marginal differences were observed for species ($p = 0.064$) (Figure S2). All these indicated that Probio87 prevented a shift in the vaginal microbial community compositional changes after 12 weeks as compared to the placebo.

3.2.3 | Core Microbiome

The core microbiome of vaginal swab samples was analyzed using a sample prevalence cutoff of 20% and relative abundance of 0.02%. A shift in core microbiome was observed in both groups

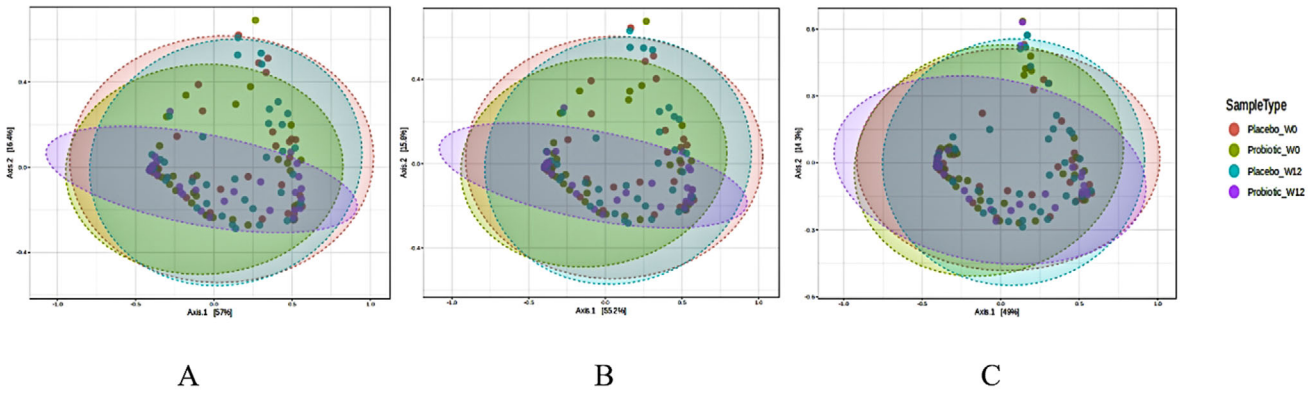

FIGURE 3 | Beta diversity was measured by Bray–Curtis dissimilarity at baseline (W0) and week 12 (W12) after *L. plantarum* Probio87 or placebo administration. PCoA as measured by PERMANOVA for (A) family, (B) genus, and (C) species. No significant differences were noted between groups at W0: family ($p = 0.259$), genus ($p = 0.142$), and species ($p = 0.165$). At W12, significant differences were observed for family ($p = 0.045$) and genus ($p = 0.037$), with marginal significance for species ($p = 0.062$). $n = 82$ (Probio87 $n = 39$, placebo $n = 43$).

over time. At the genus level, *Streptococcus* appeared in the placebo group after 12 weeks but was absent at baseline, whereas the probiotic group showed no change in core genera over time (Figure S3). *Streptococcus*, especially group B *Streptococcus*, are common in the normal vaginal flora, while overabundance occasionally causes invasive infections, including urinary tract infections in adults [32]. Correlation data showed a negative association between *Streptococcus* abundance and sexual activity occurrence (VSQ item 17; $p = 0.037$, $r = -0.257$). VSQ data are from our previous research manuscript, accepted by the journal *Beneficial Microbes* (Manuscript ID: BM-1188R1).

At the species level, *Lactobacillus jensenii* became part of the core microbiome after 12 weeks in the probiotic group, with no changes in the placebo group (Figure S4). *L. jensenii*-dominated microenvironment is associated with a healthy and stable state, with no reported transition to a diseased state in the vagina [33, 34]. Our data showed that the administration of probiotics had led to a reduced presence of vaginal pathogens such as *Streptococcus* while increasing the presence of beneficial bacteria such as *L. jensenii*.

3.2.4 | Compositional Changes and Correlation Analyses

Both alpha and beta diversity, and core microbiome evaluations indicated between-group differences in the vaginal microbiota of HPV-positive women. Our present data showed that changes occurred along different taxonomic levels. Many women do not realize the occurrence of vaginal infections, increased growth of vaginal pathogens, and/or decreased growth of beneficial vaginal bacteria, especially when symptoms do not occur. The administration of probiotics could reduce unhealthy bacterial populations while increasing the beneficial ones among HPV populations. Based on this, microbiota profiling was used to further detect the specific compositional changes in bacterial populations of the vagina.

Under phylum Firmicutes (Table S2; $p = 0.022$) and several lower taxonomic levels, the probiotic group showed increased abundances or a lesser decrease, while the placebo group showed reduced abundances or a lesser increase over 12 weeks. This was observed primarily along lower taxonomic levels of orders Lactobacillales ($p = 0.021$), families Lactobacillaceae ($p = 0.014$), genus *Lactobacillus* (0.014), and species *Phascolarctobacterium faecium* ($p = 0.041$). *Lactobacilli* are crucial for a healthy vagina, where a decreased abundance has been implicated with BV [33], while *P. faecium* are SCFA producers [35, 36], which are crucial to maintain an acidic pH in the vaginal microenvironment. The vaginal abundance of *Lactobacillus* may be highly attributed to the presence of *L. jensenii*, with a positive correlation between this genus and species ($p = 0.009$, $r = 0.288$). *Lactobacillus* species produce organic acids and other metabolites that inhibit fungal growth, thereby fostering a *Lactobacillus*-dominant microenvironment, which aligns with previous findings [37]. It also correlated negatively with vulvar dryness ($p = 0.023$, $r = -0.263$) and total VuAS score ($p = 0.004$, $r = -0.334$), indicating that vaginal lactobacilli are crucial for vulva health and combating vaginal bacterial infections. VuAS data are from our previous research manuscript accepted by the journal Beneficial Microbes (Manuscript ID: BM-1188R1).

Holdemania, protective against polycystic ovary syndrome [38] and endometriosis [39], positively correlated with SCFA producers *Lachnospiraceae* NK4A136 ($p < 0.001$, $r = 0.584$) and *P. faecium* ($p < 0.001$, $r = 0.421$), supporting pH stability. *Holdemania* and *Lachnospiraceae* NK4A136 also showed negative correlations with social interactions/life impact scores (VSQ items 12–15; $p = 0.066$, $r = -0.215$ and $p = 0.008$, $r = -0.306$), linking vaginal health to social well-being. Conversely, *Intestimonas* declines under vaginal stress, while *Catenibacterium* and *Blautia wexlerae* are associated with BV [40]. *Catenibacterium* had a marginal positive correlation with *Streptococcus* ($p = 0.080$, $r = 0.195$), linking it further to BV.

The abundance of genus *Jonquetella* increased more in the placebo group than in the probiotic group over time. Linked to BV [41] and urinary carcinoma [42], *Jonquetella* was positively correlated with vulvar dryness ($p = 0.036$, $r = 0.244$), soreness ($p = 0.034$, $r = 0.247$), and total VuAS score ($p = 0.046$, $r = 0.233$) and negatively with the SCFA producer *P. faecium* ($p = 0.030$, $r = -0.240$), suggesting a potential link between *Jonquetella*, BV, and adverse vulvar health.

In the phylum Actinobacteria, the genus *Rubrobacter* showed a slight decrease in the placebo group compared to the probiotic group over time ($p = 0.095$). While *Rubrobacter* is primarily a soil bacterium, it is also found in human breast milk [43]. In this study, vaginal *Rubrobacter* abundance positively correlated with the SCFA producer *P. faecium* ($p = 0.030$, $r = 0.240$) and sexual activities (VSQ item 17; $p < 0.001$, $r = 0.488$) and negatively with vulva soreness ($p = 0.025$, $r = -0.261$), total VuAS score ($p = 0.068$, $r = -0.213$), *Jonquetella* ($p = 0.002$, $r = -0.333$), and *Streptococcus* ($p = 0.087$, $r = -0.190$), suggesting potential benefits for women's sexual physiology and vaginal and vulvar health. *Gordonia*, common in endocervical samples [44], showed a similar trend ($p = 0.021$), with a negative correlation to vulva dryness ($p = 0.083$, $r = -0.203$) and a positive correlation with *Rubrobacter* ($p < 0.001$, $r = 0.384$), indicating a supportive role in vaginal and vulvar health.

3.3 | Vaginal Microbiota: Fungal

Although significant progress has been made in understanding the bacterial communities colonizing the vaginal microenvironment, our knowledge of the fungal populations remains limited. Much less research has focused on the fungal microbiome, leaving substantial gaps in our understanding of its role in vaginal health and diseases [7].

3.3.1 | Alpha Diversity

In addition to the Shannon and Simpson indexes, the ACE index also measures richness, taking into consideration low-abundant OTUs. At baseline, both groups showed similar Shannon indexes at levels of phylum ($p = 0.171$), class ($p = 0.188$), order ($p = 0.105$), family ($p = 0.133$), genus ($p = 0.151$), and species ($p = 0.156$) (Figure 4). However, at week 12, the placebo group showed higher Shannon indexes as compared to the probiotic group at all taxonomic levels studied ($p < 0.05$). A similar trend was observed for Simpson indexes across all taxonomic levels studied (Figure S5).

3.3.2 | Beta Diversity

PERMANOVA tests differences between groups in terms of their multivariate dispersion, while ANOSIM tests in terms of average ranks of dissimilarities between groups. In our present study, both PERMANOVA (Figure 5) and ANOSIM (Figure S6) yielded similar results, where changes over time were observed with a more prominent change occurring in the probiotic group than the placebo group. PCoA as measured by PERMANOVA for different taxonomic levels showed significant changes at baseline between groups for class ($p = 0.021$), order ($p = 0.020$), family ($p = 0.022$), and genus ($p = 0.024$), but insignificant differences between groups were observed after 12 weeks (Figure 5). Insignificant changes were observed over time or between groups for taxonomic levels of phylum and species (Figures 5A,F). The observed changes were primarily in the probiotic group, with significant shifts over time at the class (probiotic $p = 0.021$, placebo $p = 0.205$), order (probiotic $p = 0.025$, placebo $p = 0.161$), family (probiotic $p = 0.043$, placebo $p = 0.221$), and genus (probiotic $p = 0.043$, placebo $p = 0.177$) levels, unlike the placebo group. This finding suggests that Probio87 reduced compositional differences between the groups, consistent with research highlighting bacteria-fungi interactions and the potential of *Lactobacilli*-containing probiotics as a treatment and preventive option for fungal vaginal dysbiosis [45].

3.3.3 | Core Microbiome

The fungal core microbiome of vaginal swabs was analyzed with a 20% sample prevalence cut-off and a 0.01% relative abundance threshold. A shift in the core microbiome occurred in both groups over time. Over 12 weeks, core microbiome diversity increased in the placebo group, with the emergence of new genera such as *Hortaea*, *Polyporales*, *Starmerella*, *Neodevriesia*, and *Penicillium*, alongside a rise in *Candida* prevalence (Figure S7A,B). The probiotic group maintained the same number of genus types over time, with the disappearance of *Sterigmatomyces* and appearance of *Cutaneotrichosporon* after 12 weeks (Figure S7C,D).

At the species level, core microbiome diversity increased in the placebo group after 12 weeks, with new species, including *C. parapsilosis, Wallemia* sp., *Hortaea werneckii*, *Polyporales*, and *Starmerella etchellsii* appearing, while *Cladosporium delicatulum* and *Aspergillus keratitidis* disappeared (Figure S8A,B). In the probiotic group, the genus count remained stable over time. However, *C. albicans*, *Sterigmatomyces halophilus*, *Cladosporium halotolerans*, and *Malassezia furfur* disappeared, while *Malassezia globosa*, *Aspergillus*, *C. delicatulum*, and *Wallemia sp.* appeared after 12 weeks. (Figure S8C,D).

VVC is a prevalent clinical condition characterized by vaginal inflammation associated with *Candida* species. While most VVC cases are attributed to *C. albicans*, there is a growing incidence of infections caused by non-*albicans Candida* species, particularly *C. parapsilosis* [46]. After 12 weeks, the study revealed a notable increase in *C. parapsilosis* within the placebo group, while *C. albicans* were no longer detectable in the probiotic group. The correlation analysis linked the abundance of *Candida* to vulvar burning/stinging (VSQ item 2; $p = 0.045$, $r = 0.222$). *M. furfur*, though rarely linked to vaginitis, has been identified as a potential cause of vulvar folliculitis in patients on immunosuppressive therapy [47]. *M. furfur*, *H. werneckii*, *C. halotolerans*, *C. delicatulum*, *Malassezia globose*, and *Aspergillus* species are not commonly implicated in vaginal infections, but rather in other bodily infections, primarily in immunocompromised patients [48–52].

3.3.4 | Compositional Changes

Alpha and beta diversity analyses, along with core microbiome assessments, revealed notable between-group and time-based shifts in the vaginal mycobiota of HPV-positive women, indicating taxonomic changes. Many women might not notice asymptomatic infections or abnormal fungal growth. Probio87 administration reduced specific pathogenic fungi, with mycobiota profiling showing distinct changes in vaginal fungal composition.

Recent studies have revealed that in healthy women, the fungal community in the vaginal microenvironment is dominated by the

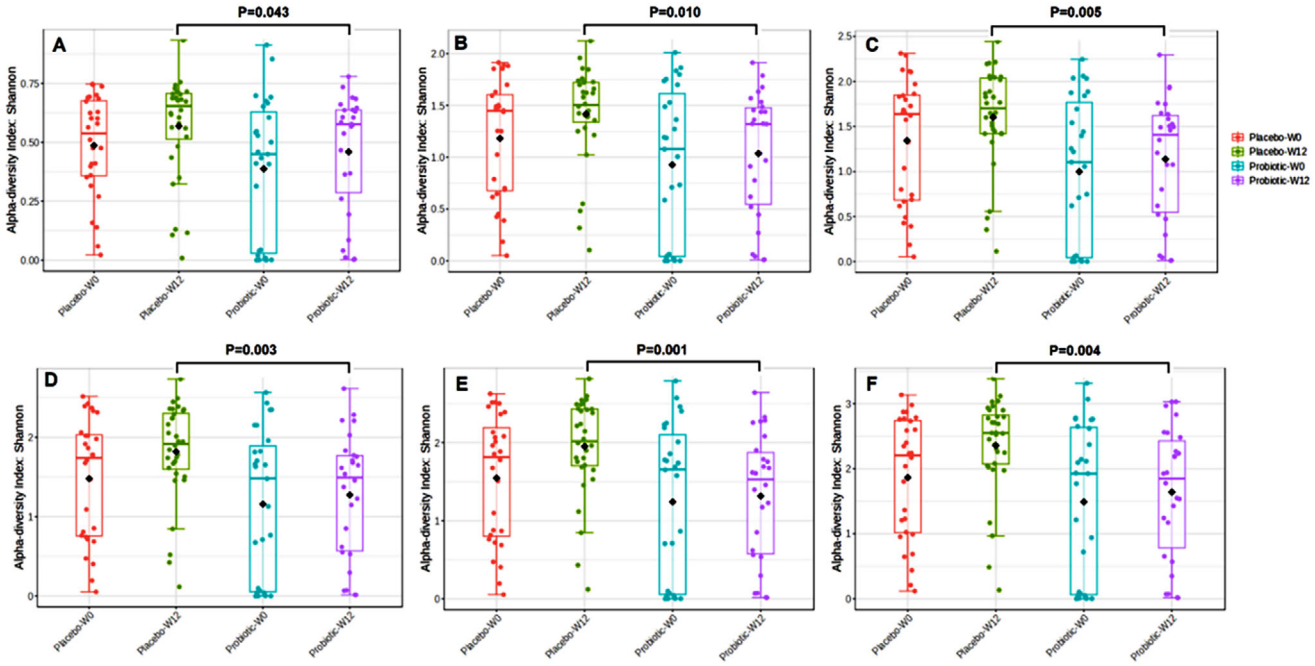

FIGURE 4 | Alpha diversity plots of vaginal fungi at baseline (week 0) and after week 12, following administration of *L. plantarum* Probio87 or placebo. Shannon index differences between groups at (A) phylum (W0, $p = 0.171$); (B) class (W0, $p = 0.188$), (C) order (W0, $p = 0.105$); (D) family (W0, $p = 0.133$); (E) genus (W0, $p = 0.151$); and (F) species (W0, $p = 0.156$). Box plots show medians (lines) and interquartile ranges (whiskers), with outliers and individual values as dots. Statistical analysis: Mann–Whitney *U* test, $n = 82$ (Probio87: $n = 39$; placebo: $n = 43$).

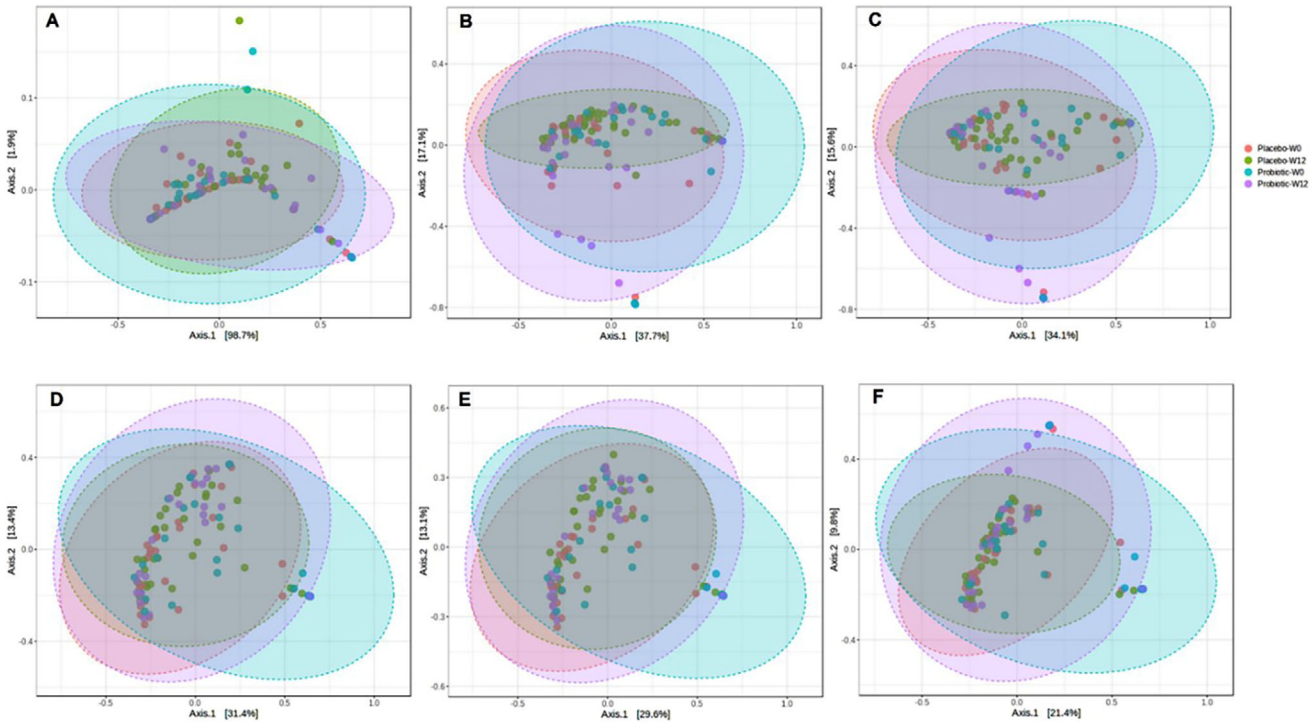

FIGURE 5 | Beta diversity as measured by the Bray–Curtis dissimilarity index are plotted at baseline (week 0) and after week-12 (W12), upon administration of probiotic *L. plantarum* Probio87 or placebo. PCoA was measured by PERMANOVA for (A) phylum, (B) class, (C) order, (D) family, (E) genus, and (F) species. Significant differences were observed at baseline (week 0) between groups: class ($p = 0.021$), order ($p = 0.020$), family ($p = 0.022$), and genus ($p = 0.024$). Significant differences were observed from the probiotic group over 12 weeks for class ($p = 0.021$), order ($p = 0.025$), family ($p = 0.043$), and genus ($p = 0.043$). $n = 82$ (Probio87 $n = 39$ and placebo $n = 43$).

phylum Ascomycota, followed by Basidiomycota. The primary orders identified within Ascomycota are Saccharomycetales, Capnodiales, Eurotiales, Pleosporales, and Helotiales [7]. The classification is similar to what we found in this study (Table S3). At the phylum level, no significant differences were observed between groups. However, at the class level, the probiotic group had significantly lower Saccharomycetes abundance than the placebo group over 12 weeks ($p = 0.002$), with similar trends at lower taxonomic levels, including the order Saccharomycetales ($p = 0.002$) and families Debaryomycetaceae (marginal, $p = 0.072$) and Phaffomycetaceae ($p = 0.023$). Correlation analysis revealed a positive association between Debaryomycetaceae abundance and difficulty showing affection (VSQ item 14; $p = 0.047$, $r = 0.220$). Significant genera differences included *Yamadazyma* ($p = 0.012$), *Komagataella* ($p = 0.037$), and *Candida* ($p = 0.011$). *Yamadazyma* abundance was positively correlated with social interactions/life impact (VSQ item 15; $p = 0.039$, $r = 0.228$) and the abundance of *Candida* ($p = 0.033$, $r = 0.236$). Some *Yamadazyma* species, typically harmless, can become opportunistic pathogens, notably *Y. triangularis*, which has emerged in clinical cases [53]. Its abundance correlated with discharge release (VSQ item 6; $p = 0.005$, $r = 0.306$) and social impact (VSQ item 15; $p = 0.019$, $r = 0.259$). *Yamadazyma triangularis* is one species noted in clinical case reports as an emerging pathogen, causing fungemia in immunocompromised patients [53]. Our correlation analysis revealed a negative association between the abundance of *Y. triangularis* and *Gordonia* ($p = 0.039$, $r = -0.228$) and a positive correlation with pathogens *Candida metapsilosis* ($p = 0.016$, $r = 0.265$) and *Penicillium* ($p = 0.013$, $r = 0.275$).

Species within the genus *Candida* represent some of humans' most significant opportunistic fungal pathogens, where frequently associated species include *C. albicans* (65.3%), *C. glabrata* (11.3%), *C. tropicalis* (7.2%), *C. parapsilosis* (6.0%), and *Candida krusei* (2.4%) [54, 55]. In the context of fungal vulvovaginal infections, *C. albicans* is consistently the most prevalent species, with studies reporting its prevalence ranging from 20.3% to 91.4% among recovered isolates [56, 57]. The abundance of *C. albicans* was positively correlated with *C. metapsilosis* in our present study ($p = 0.022$, $r = 0.252$) and positively correlated with vulvar burning or stinging symptoms (VSQ item 2; $p = 0.011$, $r = 0.280$). *C. metapsilosis* (within the *C. parapsilosis* complex) was positively correlated with vaginal discharge (VSQ item 6; $p = 0.044$, $r = 0.223$) and its social/quality of life impact (VSQ items 13 and 15; $p = 0.019$, $r = 0.260$; $p = 0.009$, $r = 0.287$). *C. oceani* also correlated with quality-of-life impact (VSQ item 15; $p < 0.001$, $r = 0.439$). Similarly, *C. parapsilosis* and *Wickerhamiella pararugosa* (formerly *Candida pararugosa*) showed positive correlations with discharge (VSQ item 6; $p = 0.044$, $r = 0.223$ and $p = 0.003$, $r = 0.326$) and quality of life (VSQ item 15, $p = 0.043$, $r = 0.224$ and $p = 0.007$, $r = 0.295$). These correlations suggest that reducing vaginal fungal infections is key to alleviating discharge symptoms, enhancing social interactions, and ultimately improving overall quality of life.

Additionally, *C. glabrata* showed a negative correlation with the abundance of *Lactobacillus salivarius* ($p < 0.001$, $r = -0.424$). *L. salivarius* is a lactic acid bacterium (LAB) commonly found in the oropharyngeal and gastrointestinal tract. Many strains can produce bacteriocins with antimicrobial properties, highlighting their potential probiotic benefits. It also supports healthy GM by improving its composition and metabolic profile, contributing to a balanced internal environment [58]. In recent years, non-*albicans Candida* species have emerged as significant pathogens. *Candida dubliniensis* has been isolated during oral candidiasis of HIV-infected individuals and from the genital tract of normal women with vaginitis [59]. *Candida haemulonii*, a rare and emerging yeast pathogen, has gained attention due to its increasing virulence and resistance to common antifungal treatments. This pathogen has a notable propensity for infecting chronic lower extremity wounds, particularly in diabetic patients [60]. *C. metapsilosis* is an opportunistic human pathogen, responsible for only a small percentage of invasive *Candida* infections [61]. The abundance of *C. metapsilosis* was positively correlated with pathogenic *Candida* species ($p = 0.026$, $r = 0.245$), *C. albicans* ($p = 0.022$, $r = 0.252$), *Candida oceani* ($p = 0.002$, $r = 0.337$), *C. parapsilosis* ($p < 0.001$, $r = 0.483$), *W. pararugosa* ($p = 0.005$, $r = 0.306$) and *Y. triangularis* ($p = 0.016$, $r = 0.265$). Additionally, *C. parapsilosis* showed a positive correlation with pathogens *Scytalidium infestans* ($p = 0.005$, $r = 0.305$), *Candida haemuloni* ($p < 0.001$, $r = 0.431$), *C. metapsilosis* ($p < 0.001$, $r = 0.483$), *C. oceani* ($p = 0.013$, $r = 0.274$), *Penicillium* ($p = 0.018$, $r = 0.260$), *Scytalidium* ($p = 0.005$, $r = 0.305$), and *Acremonium* ($p = 0.006$, $r = 0.303$). *C. oceani* was positively correlated with the abundance of *Lactobacillus iners* ($p < 0.001$, $r = 1.000$). Although *Lactobacilli* are generally beneficial for vaginal health, some species are detrimental and have been associated with symbiotic growth of vaginal pathogens, including *L. iners*, which are often associated with increased growth of pathogens involved in BV. However, the *L. jensenii*-dominated microenvironment is associated with a healthy and stable state, with no reported transition to a diseased state in the vagina [33, 34].

Within the class Sordariomycetes under phylum Ascomycota, a reduced abundance was observed over time in the probiotic group but not in the placebo group for genus *Acremonium* ($p = 0.032$). Genus *Acremonium* is frequently isolated from food and is recognized as a pathogen responsible for human diseases [62]. The abundance of *Acremonium* was positively correlated with *C. albicans* ($p = 0.011$, $r = 0.279$), *C. haemuloni* ($p = 0.023$, $r = 0.251$), *C. parapsilosis* ($p = 0.006$, $r = 0.303$), and *Acremonium sclerotigenum* ($p < 0.001$, $r = 0.442$).

In the class Dothideomycetes and other classes of Ascomycota, several orders and lower taxa differed between groups. The order Dothideales showed a marginal reduction in the probiotic group versus placebo over 12 weeks ($p = 0.1$), with similar trends in lower taxa. Dothideales include plant pathogens and endophytes like genera *Alternaria* and *Cladosporium*, which can cause opportunistic infections in immunocompromised individuals. Helotiaceae and Sordariaceae have decomposers and plant pathogens linked to respiratory issues and allergies. Helotiaceae abundance was positively correlated with vaginal discharge (VSQ item 6; $p = 0.013$, $r = 0.273$) and quality of life impact (VSQ item 15; $p = 0.038$, $r = 0.229$). Though rare, *Penicillium* can cause lung infections in weakened individuals [63], while *Talaromyces marneffei* leads to systemic infections [64]. *S. infestans* causes skin and nail infections, common in tropical areas [65]. Correlation analysis showed a positive association between *Scytalidium* and *S. infestans* abundance with vaginal discharge (VSQ item 6; $p = 0.013$, $r = 0.273$) and quality of life impact (VSQ item 15; $p = 0.038$, $r = 0.229$). *Toxicocladosporium irritans* and *A. sclerotigenum*

can cause dermatological and systemic infections, especially in immunocompromised individuals [62, 66], while *Neurospora* is generally benign [67]. *Neurospora* abundance was positively correlated with vulvar dryness (VSQ item 5; $p = 0.001$, $r = 0.371$). These findings suggest that reducing vaginal fungal infections may alleviate vaginal discharge and dryness, improve social interactions, and reduce superficial and systemic infections.

In the phylum Basidiomycota, Agaricomycetes showed the most differences between groups, with increased abundance in the placebo group and reduced abundance in the probiotic group over 12 weeks; this trend was consistent at lower taxonomic levels. Though not usually associated with human diseases, these fungi can cause opportunistic infections or allergies in immunocompromised individuals. A rare liver abscess from *S. halophilus* was reported in a boy with acute lymphoblastic leukemia [68]. Correlation analysis showed a positive association between *Sterigmatomyces* abundance and difficulty showing affection (VSQ item 14; $p = 0.04$, $r = 0.227$).

Vaginal health appears to be maintained by a stable, low-diversity fungal community without overgrowth; disruptions that allow dominance by species such as *Candida* are commonly associated with infections and clinical symptoms [69]. Our present data showed that vaginal pathogenic fungus remained in higher abundance among the placebo group as compared to the probiotic in HPV-positive women. Vaginal fungi were suppressed by vaginal bacteria such as *Lactobacillus* and *Phascolarctobacterium*, aligning with recent research demonstrating that specific probiotics and intestinal symbiotic bacteria have the capacity to prevent fungal invasion and colonization [70].

4 | Concluding Remarks

This study aimed to investigate the effects of probiotic supplementation on the vaginal microbiota of HPV-positive women, focusing on preventing and treating vaginal dysbiosis. The administration of *Lactiplantibacillus plantarum* Probio87 significantly modulated the vaginal bacterial and fungal communities over 12 weeks. The probiotic group exhibited reduced abundance of pathogenic bacteria such as *Streptococcus, Jonquetella*, and *Catenibacterium*, while beneficial bacteria like genera *Lactobacillus* and species such as *L. jensenii* and *P. faecium* increased over time, an effect not observed in the placebo group. The probiotic group also showed decreased abundance of pathogenic fungi, including *C. albicans* and *C. parapsilosis*, accompanied by increased abundance of beneficial fungi like *Malassezia globosa* over time. The results highlighted the potential of *Lactiplantibacillus plantarum* Probio87 as a therapeutic agent for restoring vaginal microbiota balance in HPV-positive women, thereby mitigating the risk of infections and complications associated with dysbiosis.

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Conflicts of Interest

All authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: mnfr70142-sup-0001-FigureS1-S8.docx. **Supporting File 2**: mnfr70142-sup-0002-TableS1-S3.docx.