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RESEARCH

Clinical and vaginal microbiota effects of oral *Lactobacillus crispatus* M247 combined with vaginal laser therapy in menopausal women with atrophic vulvovaginitis: a prospective, randomized and controlled study

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Abstract

Vaginal atrophy is a common condition in postmenopausal women, frequently associated with vaginal symptoms and alterations of the vaginal microbiota. This randomized study evaluated the clinical and microbiological effects of vaginal CO_2 laser therapy administered alone or in combination with an oral probiotic containing *L. crispatus* M247 in postmenopausal women with vulvovaginal atrophy. Women were randomized (2:1) to receive vaginal laser therapy alone (laser-only group) or laser therapy plus oral probiotic for 90 days (probiotic plus laser group). Clinical efficacy was assessed through a prespecified panel of outcomes, including vaginal pH and symptom severity assessed by Visual Analogue Scale (VAS), at baseline and during follow-up up to 90 days, with Day 90 representing the main efficacy timepoint. Vaginal microbiota composition was analyzed in a subset of participants. Both groups showed improvement over time in vaginal pH and VAS scores for vaginal dryness, dyspareunia, burning, itching, and introital pain. Compared with laser alone, the probiotic plus laser group showed lower symptom scores at Day 90 in selected clinical domains, with effect estimates generally favoring the probiotic plus laser group, although confidence intervals were relatively wide for some endpoints. P-values for individual outcomes should be interpreted as nominal in the absence of prespecified multiplicity adjustment across clinical domains. For vaginal pH, both groups showed a decrease over time, but the group × time interaction was not statistically significant, indicating no clear differential temporal response. Microbiota analysis showed an increase in the relative abundance of L. crispatus and a concomitant decrease in L. iners only in the probiotic plus laser group, suggesting a shift toward a more favorable vaginal microbial profile. Overall, vaginal CO_2 laser therapy was associated with clinical improvement, and adjunctive treatment with *L. crispatus* M247 was associated with additional benefits across selected clinical and microbiological outcomes; however, confidence intervals for several clinical estimates were relatively wide, supporting cautious interpretation and confirmation in larger blinded studies.

Keywords Genitourinary syndrome · Probiotic · Vaginal pH · Dyspareunia · Laser CO_2

Introduction

Genitourinary syndrome of menopause (GSM) encompasses a constellation of symptoms affecting the lower genital tract, including vulvovaginal atrophy (VVA), as well as lower urinary tract symptoms (LUTS), resulting from the progressive decline in estrogen levels occurring during the menopausal transition [1]. Common manifestations of GSM within the urogenital tract include vaginal irritation, pruritus, burning sensation, dryness, dyspareunia, and a range of urinary complaints [2]. Beyond physical discomfort, these symptoms exert a significant negative impact on sexual function and overall quality of life in affected women [3]. Consequently, women experiencing

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GSM-related symptoms require effective therapeutic interventions aimed at symptom relief and restoration of a vaginal environment resembling that of the premenopausal state [3]. Several local treatment options are currently available for the management of GSM, targeting either symptom alleviation or the restoration of normal urogenital physiology. When non-hormonal approaches such as vaginal moisturizers and lubricants fail to adequately control symptoms, local estrogen therapy represents the standard treatment for women with vulvovaginal manifestations of GSM [4, 5]. In cases where estrogen therapy is ineffective, contraindicated, or poorly tolerated, vaginal laser therapy has emerged as a non-pharmacological second-line alternative, particularly relevant for women who are non-responsive or non-compliant and for those with hormone-related contraindications, including breast cancer survivors [6]. The vaginal microbiota is commonly classified into five Community State Types (CST I–V) based on bacterial diversity and the predominance of *Lactobacillus* species. Microbial communities characterized by low diversity and dominance of *L. crispatus* (CST I) are associated with a reduced obstetric and gynecological risk. Conversely, communities displaying high bacterial richness and limited *Lactobacillus* spp. dominance (CST IV) are frequently associated with vaginal discomfort and a higher prevalence of gynecological and obstetric disorders [7]. Recognition of *L. crispatus* as the species most consistently associated with vaginal health across different ethnic groups has driven the development of probiotic formulations containing this microorganism for both vaginal administration and oral administration with vaginal targeting [8]. The rationale for the use of *L. crispatus*-based probiotics lies in the premise that successful vaginal colonization by strains of this species may facilitate the re-establishment of eubiosis, thereby reducing obstetric and gynecological risk, including in postmenopausal women [9–11]. Current evidence indicates that fractional micro-ablative carbon dioxide (CO_2) laser therapy can alleviate GSM symptoms through mechanisms related to vaginal tissue remodeling [12]. Specifically, consistent short-term improvements have been reported for vaginal dryness, dyspareunia, itching and burning, sexual function, dysuria, urinary frequency and urgency, and urinary incontinence in studies evaluating the efficacy of CO_2 laser therapy [12]. However, despite numerous early-phase studies reporting significant short-term symptomatic benefits following CO_2 laser treatment, the strength of this evidence has been challenged by recent randomized controlled trials showing no statistically significant differences between laser and sham procedures, thereby questioning earlier assumptions regarding its clinical effectiveness [13]. Given that previous investigations have primarily focused on symptom-based outcomes, with limited assessment of vaginal microbiota changes beyond the genus level of *Lactobacillus*, the present study, prospective, randomized and controlled, was designed to evaluate the effects of vaginal laser therapy on vaginal microbial communities at the CST level, with or without the adjunctive use of an oral probiotic containing *L. crispatus* M247 [14].

Materials and methods

Study ethics and involved centres

This study is a prospective, randomized, controlled, open-label, non-profit, multicenter clinical trial conducted at Villa Margherita Hospital (Rome, Italy) and at the Hospital of the State of the Republic of San Marino (San Marino). The study was conducted in accordance with the principles of Good Clinical Practice (GCP) and the Declaration of Helsinki. The trial was registered on ClinicalTrials.gov (Identifier: NCT06978907; date of registration: May 11, 2025, prior to patient enrollment). Ethical approval for the study protocol was obtained before study initiation from the Ethics Committee of the University of Urbino "Carlo Bo" (Italy), as documented in the Minutes of Session No. 93 of April 24, 2025. All participants provided written informed consent prior to inclusion in the study. No identifiable personal data or images are reported.

Study design

The study flow chart is shown in Fig. 1. Of the 109 postmenopausal women assessed for eligibility during the study period, 75 met the enrollment criteria and were included in the study. Randomization was performed using a computer-generated allocation sequence with a predefined 2:1 ratio in favor of the laser-only group. No stratification by center or baseline characteristics was applied, and no formal blocking procedure was used. After eligibility had been confirmed and written informed consent had been obtained, participants were assigned sequentially according to the pre-established randomization list. Participant screening and enrollment were performed at the participating centers, while treatment assignment followed the previously generated allocation sequence. Because of the pragmatic open-label design of the study, no additional formal allocation concealment procedure was implemented. As 4 patients allocated to the probiotic plus laser group and 3 allocated to the laser-only group were lost to follow-up, the clinical evaluation was performed in 21 women

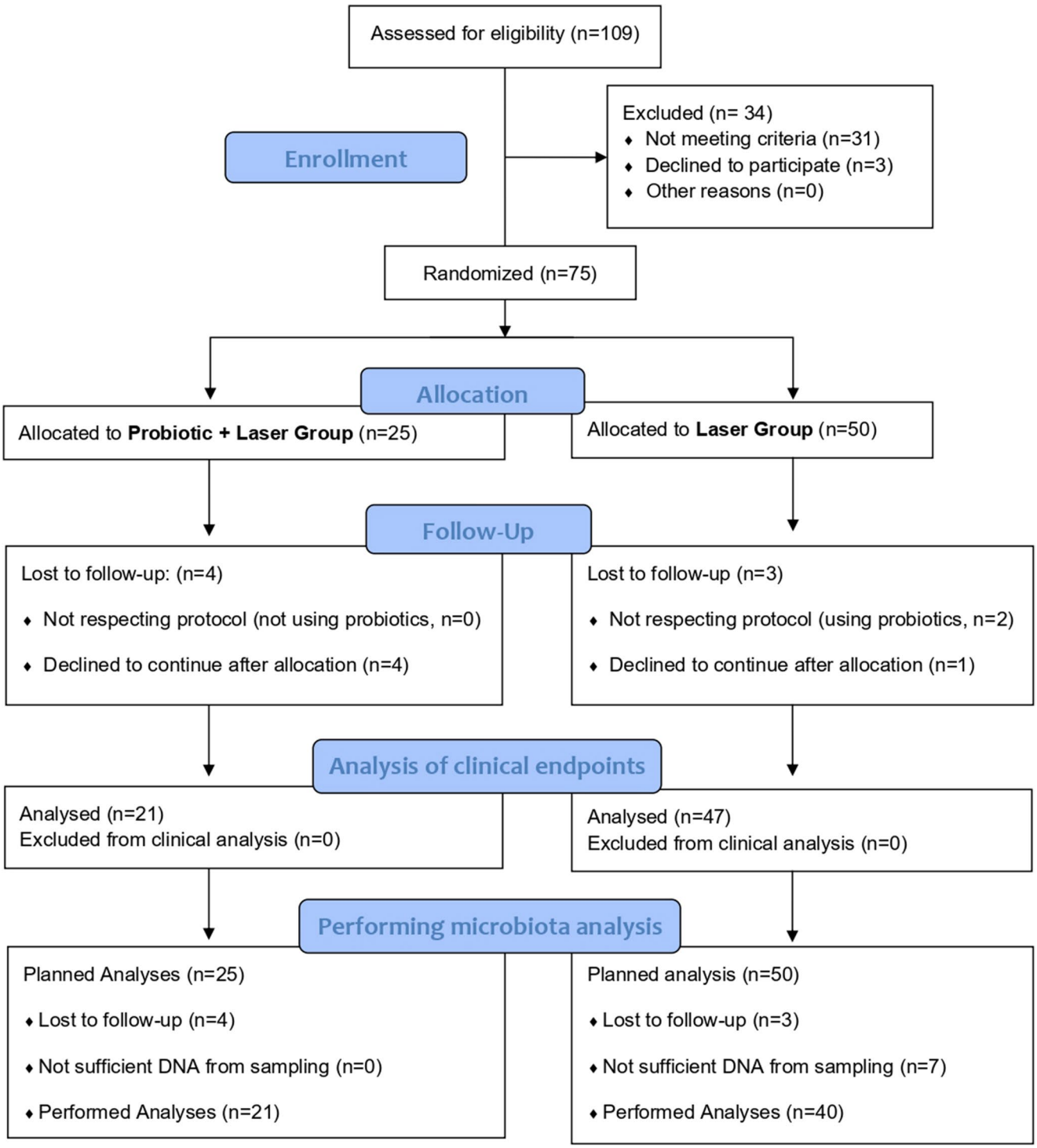

Fig. 1 Flow chart of the study

in the probiotic group and in 47 women in the laser-only group. Due to insufficient DNA yield from vaginal sampling, microbiota analysis was performed in 40 women in the laser-only group. In all women of the probiotic plus laser group, vaginal sampling yielded sufficient DNA for microbiota analysis.

Inclusion and exclusion criteria

The inclusion criteria were: (1) postmenopausal women, spontaneous or induced with a lack of menstrual periods for >12 months and a diagnosis of VVA and experiencing related symptoms (vaginal dryness, introital pain and

dyspareunia, itching, burning, bleeding during intercourse); because dyspareunia was one of the main reasons for clinical referral and study participation, all enrolled women were sexually active, or attempting vaginal intercourse, during the study period; (2) postmenopausal women with GSM-related symptoms (urinary urgency, increased urinary frequency, nocturia, recurrent cystitis, and postcoital cystitis); (3) postmenopausal women who had not responded to previous local hormonal replacement therapy (HRT) or who had contraindications to local and/or systemic HRT; (4) postmenopausal women with previous negative PAP test performed within one year of enrollment. The exclusion criteria were: (1) preneoplastic or neoplastic lesions of the cervix, vagina, and vulva; (2) presence of active genital and/or urinary tract infection; (3) dermatological contraindications, relative or absolute, to laser use; (4) current systemic or local hormone therapy (6) Neurological and/or psychiatric disorders; (6) chronic systemic autoimmune or metabolic diseases; (7) use of probiotics in the 4 weeks before enrolment.

Treatments

Patients of the probiotic group were administered with the probiotic strain *L. crispatus* M247 (LMGP-23257). Treatment was once a day, after breakfast, by dissolving the preparation powder in water or directly into the oral cavity, for three continuous months. Each probiotic sachet contained no fewer than 20 billion colony forming units (CFU) bacteria. The product (Crispact®, Pharmextracta SpA, Pontenure, Piacenza, Italy; notified to the Italian Health Authorities in 2019 with the notification number 115450) was manufactured by Alfa-Omega Srl (Copparo, FE; Italy). Enrolled women in both the probiotic and control groups were treated with vaginal laser therapy using the MonaLisa Touch® fractional CO_2 laser (DEKA Srl, Calenzano, FI, Italy). Three microfractionated CO_2 laser treatment sessions were performed, with a minimum interval of 4 weeks between two consecutive sessions. Laser settings were selected according to the anatomical target area. For the endovaginal treatment, parameters were: power 40 W, dwell time 1000 µs, spacing 1000 µm, SmartStack 2, DEKA pulse, corresponding to a fluence of approximately 113 J/cm² and an average ablation depth of about 250 µm. For the vaginal introitus, parameters were: power 25–30 W, dwell time 1000 µs, spacing 1000 µm, SmartStack 1, DEKA pulse, corresponding to a fluence of approximately 35–42 J/cm² and an average ablation depth of 75–100 µm. For the vulvar, clitoral, and perianal areas, when treated, parameters were: power 20–25 W, dwell time 1000 µs, spacing 1000 µm, SmartStack 1, DEKA pulse, again corresponding to a fluence of approximately 35–42 J/cm² and an average ablation depth of 75–100 µm. The MonaLisa Touch technique was delivered using two handpieces according to the treatment area. The 360° device allowed circumferential delivery of laser energy within the vaginal canal and was used to treat the vaginal mucosa from the fornices to the lower third of the vagina, with uniform coverage of the vaginal walls. The 0° device emitted the laser in a frontal and directional manner and was used for more focal treatment of the vaginal introitus, vulvar vestibule, scarred or atrophic areas, and other external genital sites when clinically indicated. No pre-treatment analgesia and no vaginal gel were used before the procedure. However, an anesthetic cream was applied immediately after treatment to reduce the initial inflammatory reaction. All procedures were performed by experienced physicians with long-standing clinical expertise in vaginal and vulvar laser microfractionation using the MonaLisa Touch technique, in order to maintain procedural consistency across centers.

Study outcomes

The primary objective of the study was to evaluate the overall clinical efficacy and safety of adding oral Lactobacillus crispatus M247 to vaginal fractional CO_2 laser treatment in postmenopausal women with GSM/VVA. To operationalize this objective, clinical efficacy was assessed through a prespecified panel of outcomes, including vaginal pH, dyspareunia, introital pain, vaginal dryness, vaginal itching, vaginal burning, Patients' Global Expectation (PGE), and Patients' Global Satisfaction (PGS). Vaginal itching was included among the longitudinal symptom outcomes assessed during follow-up, whereas urinary symptoms were not prespecified as repeated study outcomes and were recorded only as part of the broader clinical characterization when available. The main efficacy timepoint was Day 90 (T90), while assessments performed at T30 and T60 were considered supportive longitudinal evaluations. Safety was assessed through adverse events recorded during the study period. Vaginal microbiota composition, including community state type distribution, richness, and the relative abundance of Lactobacillus species, was evaluated as a secondary outcome.

Objective and subjective assessment measures

Vaginal pH was measured using color-fixed indicator sticks ranging from pH 0–14 (pH ≥ 5 indicated vaginal atrophy, pH < 5 indicated good estrogenization) [15]. Each patient in both groups completed at home the Visual Analogue Scale (VAS) for vaginal atrophy symptoms, together with the Patients' Global Expectation (PGE) and Patients' Global Satisfaction (PGS) questionnaires, and submitted their responses to the collaborating researchers without possibility of alteration [16]. To minimize investigator-related

influence on subjective reporting, the same written questionnaires, response format, and symptom anchors were used consistently at each study visit. No formal blinded outcome assessment for symptom endpoints and no scripted counseling procedure were implemented. Vaginal dryness, itching, burning, and dyspareunia were assessed using a self-administered 10-point VAS ranging from 0 (absence of symptoms) to 10 (maximum symptom severity). The same symptom-specific VAS format was used at each study visit to ensure consistency of administration over time. Scores < 4 indicated mild symptoms, scores ≥ 4 to < 8 moderate symptoms, and ≥ 8 severe symptoms. This pragmatic approach was chosen because it is simple, repeatable, and suitable for longitudinal symptom monitoring in routine clinical settings; however, it does not represent a formally validated GSM/VVA-specific patient-reported outcome instrument. Dyspareunia was assessed in a population of women who were all sexually active, or attempting vaginal intercourse, during the study period, as confirmed by the participating gynecologists at enrollment. No participants were excluded from dyspareunia analyses because of sexual inactivity. However, the frequency of sexual activity was not quantitatively recorded as a longitudinal study variable. Pain at the vaginal introitus was assessed using a VAS ranging from 0 (absence of symptoms) to 10 (severe symptoms) with (0) indicating no pain, (1–3) mild pain, (4–6) moderate pain, and (7–10) severe pain [16]. PGE and PGS with treatment were assessed using a Likert-type scale, a widely used ordinal self-report instrument for capturing subjective attitudes and perceptions in clinical research, ranging from 0 (no expectation; dissatisfied) to 10 (very high expectation; extremely satisfied) [17]. Adverse events (AE) were assessed based on severity and resulting disability. AE were self-reported by patients and picked up at each visit, and by phone call when needed, throughout the study. AEs were classified according to Common Terminology Criteria for Adverse Events (CTCAE) v5.0 [18].

Vaginal microbiota analysis and CST classification

For the purposes of this study, vaginal swab samples were collected at baseline (T = 0) and at follow-up (T = 90) from 68 women (47 in the laser-only group and 21 in the probiotic plus laser group). Swabs were placed in sterile tubes containing 1 mL of DNA/RNA Shield (Zymo Research) and stored until bacterial DNA extraction. Due to insufficient DNA yield in samples from 7 women in the laser-only group, microbiota analyses were performed on 61 women (40 in the laser-only group and 21 in the probiotic plus laser group). Vaginal samples were subjected to DNA extraction using the ZymoBIOMICS DNA Miniprep Kit following the manufacturer's instructions (Zymo Research, Irvine, CA, USA). Partial 16 S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio_Uni/Probio_Rev, targeting the V3 region of the 16 S rRNA gene sequence [19]. Illumina adapter overhang nucleotide sequences were added to the partial 16 S rRNA gene-specific amplicons, which were further processed employing the 16 S Metagenomic Sequencing Library Preparation Protocol (Part #15044223 Rev. B - Illumina, San Diego, CA, USA). Amplifications were carried out using a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The integrity of the PCR amplicons was analyzed by electrophoresis on a 2200 TapeStation Instrument (Agilent Technologies, Santa Clara, CA, USA). The DNA products obtained following PCR-mediated amplification of the 16 S rRNA gene sequences were purified by a magnetic purification step involving Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. The DNA concentration of the amplified sequence library was determined by a fluorometric Qubit quantification system (Life Technologies – Thermo Fisher Scientific Inc., Waltham, MA, USA). Amplicons were diluted to a concentration of 4 nM, and 5 µL quantities of each diluted DNA amplicon sample were mixed to prepare the pooled final library. Sequencing was performed using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals. Following sequencing, the.fastq files were processed using a custom script based on the QIIME software suite [20]. Paired-end read pairs were assembled to reconstruct the complete Probio_Uni/Probio_Rev amplicons. Quality control retained sequences with a length between 140 and 400 bp and a mean sequence quality score > 20, while sequences with homopolymers > 7 bp and mismatched primers were omitted. In order to calculate downstream diversity measures, 16 S rRNA operational taxonomic units (OTUs) were defined at 100% sequence homology (also called ASVs) using DADA2 [21]. OTUs not encompassing at least two sequences of the same sample were removed. Notably, this approach allows highly distinctive taxonomic classification at single nucleotide accuracy [22]. All reads were classified to the lowest possible taxonomic rank using QIIME2 [23, 24]. and a reference dataset from the SILVA database [25]. Biodiversity within a given sample (α-diversity) was calculated using the OTU number. Regarding *Lactobacillus* partial ITS sequences were amplified from extracted DNA using the primer pair Probio-lac_Uni/Probio-lac_Rev, which targets the spacer region between the 16 S rRNA and the 23 S rRNA genes within the rRNA locus [26]. Illumina adapter overhang nucleotide sequences were added to the partial ITS amplicons, which were further processed employing the 16 S Metagenomic Sequencing Library Preparation Protocol (Part#15044223 Rev. B - Illumina). PCR amplification as well as library

preparation were carried out as described above for the 16 S rRNA microbial profiling analyses. Following sequencing, the.fastq files were processed using a custom script based on the QIIME software suite. Paired-end read pairs were assembled to reconstruct the complete Probio-lac_Uni/Probio-lac_Rev amplicons. Quality control retained sequences with a length between 100 and 400 bp and a mean sequence quality score of > 20, while sequences with homopolymers > 7 bp in length and mismatched primers were removed. ITS OTUs were defined at 100% sequence homology using UCLUST [24]. All reads were classified to the lowest possible taxonomic rank using QIIME2 [23, 24]. and a reference dataset, consisting of an updated version of the *Lactobacillus* ITS database [26]. CST classification was performed according to the *Lactobacillus* dominance [27].

Sample size calculation and post hoc analysis

The sample size was calculated assuming an expected 30% improvement in Visual Analogue Scale (VAS) scores for vaginal atrophy–related symptoms in the probiotic plus laser group compared with the laser-only group. Based on an 80% statistical power and a two-sided alpha level of 0.05, a minimum of 21 women per group was estimated to be sufficient to detect clinically meaningful between-group differences in symptom severity [28]. To account for an anticipated dropout rate of approximately 15% over the 3-month study period, the planned sample size was increased to a minimum of 50 women overall. A predefined 2:1 randomization ratio in favor of the laser-only group was adopted to enhance the precision of outcome estimates in the control arm while maintaining an adequately sized probiotic plus laser group for comparative analyses. A total of 75 women were enrolled, exceeding the planned sample size and comparable to or larger than that reported in similar studies [29, 30]. After accounting for loss to follow-up, the final analyzed sample consisted of 47 women in the laser-only group and 21 in the probiotic plus laser group. Given the observed sample size and effect magnitude, the study was adequately powered to detect moderate-to-large differences in continuous clinical outcomes, whereas smaller effects may have remained underpowered.

Statistical analysis

Statistical analyses were performed to compare baseline characteristics, clinical outcomes, and vaginal microbiota parameters between the two study groups. Continuous variables were summarized as mean ± standard deviation or median and interquartile range (IQR), as appropriate, while categorical variables were reported as absolute and relative frequencies. Baseline comparability between groups was assessed using the Wilcoxon–Mann–Whitney test for continuous variables and Fisher's exact test or Pearson's chi-square test for categorical variables, depending on expected cell counts. Changes in vaginal pH over time were analyzed using a longitudinal mixed-effects model with subject-specific random intercepts to account for repeated measures. Fixed effects included time, treatment group, and their interaction. Least-squares means were estimated and post hoc comparisons were performed using Tukey's honestly significant difference (HSD) test. Because the Reviewer raised concern regarding possible baseline imbalance, pH results were additionally reported as change from baseline to T90, and interpretation of treatment-related differences was based primarily on the group × time interaction and on the baseline-to-T90 interaction contrast derived from the mixed model, which was reported together with its 95% confidence interval. Longitudinal changes in clinical symptom severity assessed by Visual Analogue Scale (VAS) scores were analyzed using linear mixed-effects models with subject-specific random intercepts, including time, group, and time × group interaction as fixed effects. When required by model assumptions, outcomes were natural log–transformed prior to analysis. No zero values were observed for VAS measures; therefore, log-transformation did not require the use of offsets. Least squares means were used to estimate marginal means across time points and groups, and post hoc comparisons were performed using Tukey's HSD procedure. Within-group paired comparisons between baseline (T = 0) and follow-up (T = 90) were additionally evaluated using the Wilcoxon signed-rank test, while between-group comparisons at each time point were conducted using the Wilcoxon–Mann–Whitney test for non-normally distributed variables. These non-parametric analyses were performed as sensitivity analyses to support the findings obtained from mixed-effects models. In addition, post hoc covariate-adjusted sensitivity analyses were performed for key endpoints at T = 90 using linear models with treatment group as the main factor and including the baseline value of the endpoint, age, and current smoking status as covariates. These analyses were considered supportive and were used to assess the robustness of the main findings to clinically relevant baseline imbalances. For dyspareunia, all women included in the clinical analyses were considered eligible for evaluation because the enrolled population was sexually active, or attempting vaginal intercourse, during the study period; therefore, no observations were excluded or coded as missing because of sexual inactivity. Sexual activity frequency was not analyzed as a separate longitudinal covariate. Vaginal microbiota richness, expressed as amplicon sequence variant (ASV) counts, was analyzed using mixed-design (split-plot) analysis of variance to assess the main effects of time, group, and their interaction.

Relative abundances of vaginal bacterial *taxa* were analyzed according to their distributional characteristics. For dominant Lactobacillus species and other *taxa* showing skewed or zero-inflated distributions, within-group comparisons between baseline (T = 0) and follow-up (T = 90) were performed using the Wilcoxon signed-rank test, while between-group comparisons at each time point were conducted using the Wilcoxon–Mann–Whitney test. When distributional assumptions were met, longitudinal analyses of relative abundances were additionally performed using linear mixed-effects models including time, group, and time × group interaction as fixed effects. In mixed-effects models, the probiotic plus laser group and T = 90 were specified as reference categories. The study was designed to evaluate the overall clinical efficacy and safety profile of adjunctive oral *L. crispatus* M247 during vaginal CO_2 laser therapy rather than to test a single symptom-specific confirmatory endpoint. Accordingly, clinical efficacy was assessed through a prespecified panel of related outcomes reflecting VVA/GSM severity, including vaginal pH and symptom-specific VAS scores, while T = 90 was considered the main efficacy timepoint. Repeated assessments at T = 30 and T = 60 were used to describe the longitudinal trajectory of response. Because no formal multiplicity adjustment was prespecified across the different clinical and microbiological domains, p-values for individual analyses are reported as nominal and should be interpreted as supportive/exploratory. Emphasis was therefore placed on the direction, magnitude, and consistency of effects across endpoints rather than on isolated statistical significance. To complement p-value–based inference in view of the modest sample size, key clinical outcomes at T90 were additionally summarized in the main text by between-group mean differences with 95% confidence intervals and standardized effect sizes (Hedges' g), calculated on complete-case data and reported as descriptive measures of effect magnitude and precision. All statistical tests were two-sided, and a p-value < 0.05 was considered statistically significant. Analyses were conducted using JMP version 10 (SAS Institute, Cary, NC, USA).

Results

Study flow chart and descriptive features of the analyzed patients

As shown in Fig. 1, after assessing for eligibility 109 patients, 31 of the them were excluded according to the inclusion and exclusion criteria and 3 declined to participate. After randomization and allocation, 5 women (4 of the probiotic and laser group and 1 of the laser group) left the study on their own choice before the treatment started. Two women of the laser group were then excluded as they declared to have used probiotics. Out of the 68 patients left (21 belonging to the probiotic and laser group and 47 belonging to the laser one), no others abandoned the study during its course. Therefore, all the results shown refers to the 68 patients completing the study. Clinical efficacy was assessed at T = 30, T = 60, and T = 90, with T = 90 considered the main efficacy timepoint according to the prespecified analytical framework. As shown in Table 1 the two groups demonstrated no significant differences according to the all considered features but age, smoke habit, mood swings/insomnia, and bowel discomfort. Although randomization was applied, some baseline differences were observed, likely due to the modest sample size and the 2:1 allocation ratio. Importantly, all factors associated with worse symptom burden were more prevalent in the probiotic plus laser group, which would be expected to bias results toward the null; therefore, any additional improvement observed in this group is unlikely to be explained by baseline advantages alone.

Clinical efficacy outcomes

In line with the predefined analytical framework, clinical efficacy was evaluated across a prespecified panel of related outcomes, with T = 90 considered the main efficacy timepoint. To assess robustness against baseline imbalances, post hoc covariate-adjusted sensitivity analyses were also performed at T = 90 for key endpoints, including the baseline value of the endpoint, age, and current smoking status as covariates. These analyses yielded estimates that were directionally consistent with the main models. The adjusted between-group effect was −0.11 pH units (95% CI −0.42 to 0.19) for vaginal pH, −0.61 VAS points (95% CI −1.65 to 0.43) for vaginal dryness, −1.19 VAS points (95% CI −2.25 to −0.13) for vaginal burning, and −0.57 VAS points (95% CI −1.75 to 0.62) for introital pain, all favoring the probiotic plus laser group. Results for the individual clinical domains are presented below as supportive/exploratory components of the overall efficacy profile. To complement p-value–based inference, between-group mean differences at T90 with 95% confidence intervals and standardized effect sizes for key clinical outcomes are reported in the main text. Overall, these estimates generally favored the probiotic plus laser group for selected symptom outcomes, but confidence intervals were relatively wide for several domains, indicating limited precision.

Effect of treatments on vaginal pH

The least-squares means plot shown in Fig. 2 indicates that, at baseline (T = 0), vaginal pH was numerically lower in

Table 1 Descriptive features of the analyzed patients

	Probiotic + laser group (N=21)	Laser group (N=47)	Statistics
Age (years)	57.7 ± 5.6	53.7 ± 5.7	p=0.0081
Ethnic group	Caucasian: 21	Caucasian: 47	n. s.
Menarche (years)	12.3 ± 2.4	12.6 ± 2.8	n. s.
Education	Degree: 12 Diploma: 8 Other: 1	Degree: 27 Diploma: 18 Other: 2	n. s.
Socioeconomic level	High: 16 Medium: 4 Low: 1	High: 36 Medium: 9 Low: 2	n. s.
Alcohol	No: 20 Yes: 1	No: 39 Yes: 8	n. s.
EP pill (5 years before menopause)	Yes: 4 No: 17	Yes: 9 No: 38	n. s.
Smoking	Yes: 6 No: 15 Former: 0	Yes: 2 No: 44 Former:1	p=0.0138
BMI (kg/m^2)	23.6 ± 4.1	23.5 ± 2.8	n. s.
Menopause	Yes: 21 Spontaneous: 20 Induced: 1	Yes: 47 Spontaneous: 46 Induced:1	n. s.
Age of menopause	49.1 ± 4.1	50.7 ± 3.6	n. s.
HPV	Yes: 19 No: 2	Yes: 40 No: 7	n. s.
Antibiotics (last 6 months)	Yes: 2 No: 19	Yes: 7 No: 40	n. s.
Nutrition style	Gluten-free: 1 Low FODMAPs: 0 Mediterranean: 1 Vegetarian/Vegan: 0 Western: 19	Gluten-free: 2 Low FODMAPs: 1 Mediterranean: 13 Vegetarian/Vegan: 1 Western: 30	n. s.
Operative birth	Yes: 0 No: 21	Yes: 2 No: 45	n. s.
Spontaneous birth (number)	0: 9 1: 4 2: 6 3: 1 4: 1	0: 20 1: 13 2: 10 3: 4 4: 0	n. s.
Cesarean section (number)	0: 19 1: 1 2: 1 3: 0	0: 30 1: 9 2: 7 3: 1	n. s.
Mood swings Insomnia	Yes: 14 No: 7	Yes: 21 No: 26	p=0.0001
Vaginal lichen	Yes: 2 No: 19	Yes: 2 No: 45	n. s.
Bowel discomfort (History of)	Yes: 16 No: 5	Yes: 22 No: 25	p=0.0056
Perineal pain	Yes: 0 No: 21	Yes: 0 No: 47	n. s.
Recurrent cystitis (History of)	Yes: 2 No: 19	Yes: 3 No: 44	n. s.
Uterine prolapse	Yes: 5 No: 16	Yes: 9 No: 38	n. s.
Cancer (History of)	Yes: 1 No: 20	Yes: 7 No: 40	n. s.
Comorbidity	Yes: 10 No: 11	Yes: 24 No: 23	n. s.

Table 1 (continued)

	Probiotic + laser group (N=21)	Laser group (N=47)	Statistics
Comorbidity (Type of)	Metabolic: 4 Autoimmune: 1 Cardiovascular: 3 Hormonal: 2	Metabolic: 10 Autoimmune: 3 Cardiovascular: 7 Hormonal: 4	n. s.
Gynecological symptoms	Vaginal: 8 Clitoral: 2 Vestibulo-vulvar: 2 Mixed: 9	Vaginal: 17 Clitoral: 4 Vestibulo-vulvar: 5 Mixed: 21	n. s.

Age, Menarche, and BMI (Body Mass Index) are shown as Mean ± Standard Deviation

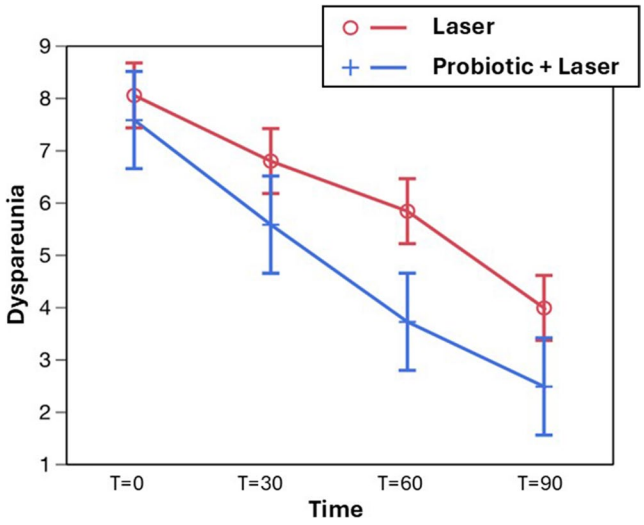

Fig. 2 Effect of treatments on vaginal pH. Least-squares means ± standard error are shown for the laser-only and probiotic plus laser groups at each time point. Vaginal pH was numerically lower in the probiotic plus laser group throughout follow-up. However, the baseline Tukey comparison was not statistically significant (Supplementary File 1), and the longitudinal mixed-effects model showed no significant group × time interaction (p = 0.89380; Supplementary File 2), indicating that the between-group separation remained essentially stable over time rather than reflecting a differential temporal response to treatment

the probiotic plus laser group than in the laser-only group (mean pH: 5.65 vs. 6.52, respectively). However, the Tukey post-hoc comparison did not indicate a statistically significant difference between groups at baseline (Supplementary File 1). Over time, both groups exhibited a reduction in vaginal pH. In the laser-only group, vaginal pH decreased from 6.52 at T=0 to 6.20 at T=90 (change: −0.32). In the probiotic plus laser group, vaginal pH decreased from 5.65 at T=0 to 5.18 at T=90 (change: −0.47). Thus, the probiotic plus laser group showed a numerically greater reduction from baseline, although the absolute difference in change was modest (−0.15 pH units). Analysis of variance based on the longitudinal mixed-effects model revealed a highly significant main effect of treatment group (p=0.00004), whereas neither the main effect of time (p=0.17076) nor the group × time interaction (p=0.89380) reached statistical significance (Supplementary File 2). This indicates that the between-group separation in pH remained essentially stable over time and does not support a differential temporal treatment response. Consistently, the model-based baseline-to-T90 interaction contrast was small (estimate −0.0809, SE 0.1224, 95% CI −0.3218 to 0.1600), suggesting that the numerical baseline difference did not materially affect the inference on pH evolution over time (Supplementary File 3). Overall, these findings support a cautious interpretation: the probiotic plus laser group maintained lower pH values throughout follow-up, but the longitudinal analysis does not demonstrate a significantly greater treatment-related reduction in pH over time compared with laser alone.

Effect of treatments on dyspareunia

Dyspareunia was evaluated in the full clinical cohort, as all enrolled women were sexually active, or attempting vaginal intercourse, during the study period. Both treatments were associated with a progressive reduction in dyspareunia severity over the study period, as assessed by Visual Analogue Scale (VAS) scores (Fig. 3). Least squares mean VAS scores decreased from 8.04 at baseline (T=0) to 3.98 at T=90 in the laser-only group, and from 7.57 at baseline to 2.48 at T=90 in the probiotic plus laser group (Supplementary File 4). At intermediate time points, mean dyspareunia scores were 6.79 (laser) and 5.57 (probiotic plus laser) at T=30, and 5.83 (laser) and 3.71 (probiotic plus laser) at T=60 (Supplementary File 4). Post-hoc comparisons using Tukey's test indicated that, starting from T=60, dyspareunia scores in the probiotic plus laser group were significantly lower than those observed in the laser-only group (Supplementary File 4). Consistently, mixed-effects model analysis demonstrated a highly significant effect of time (p<0.0001), a significant main effect of group (p=0.00572), and a significant group × time interaction (p=0.01959), supporting a differential temporal response between treatments (Supplementary File 5). Parameter estimates from the linear mixed-effects model (with the probiotic plus laser group and T=90 as reference categories) further corroborated these findings:

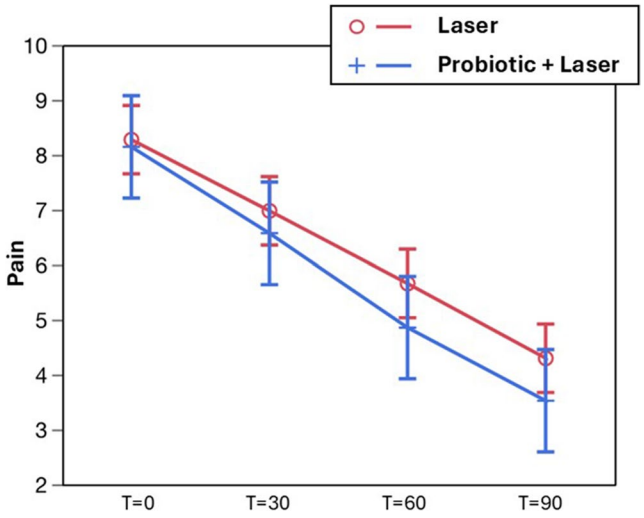

Fig. 3 Effect of treatments on dyspareunia. Mean Visual Analogue Scale (VAS) scores are shown for the laser-only and probiotic plus laser groups. Dyspareunia was evaluated in the full clinical cohort, as all enrolled women were sexually active, or attempting vaginal intercourse, during the study period. Both treatments resulted in a reduction of dyspareunia over time. From T=60 onward, the probiotic plus laser group showed lower VAS scores compared with the laser-only group, as supported by the mixed-effects model (Supplementary Files 5 and 6) and Tukey post-hoc comparisons (Supplementary File 4)

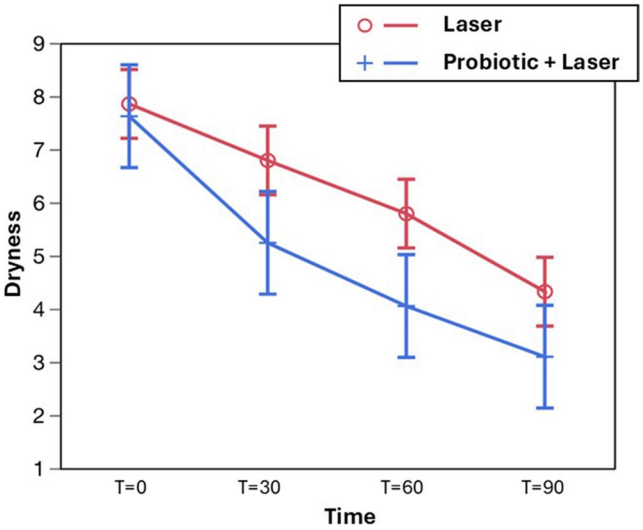

Fig. 4 Effect of treatments on introital pain. Least squares mean Visual Analogue Scale (VAS) scores (0–10) are shown for the laser-only and probiotic plus laser groups at baseline (T=0) and at follow-up time points (T=30, T=60, and T=90 days). Both treatments were associated with a progressive reduction in introital pain severity over time. No statistically significant differences between groups were observed at any time point, while within-group reductions over time were supported by Tukey HSD post-hoc comparisons (Supplementary File 7) and mixed-effects model analyses (Supplementary Files 8 and 9)

the group effect at the reference time point was significant (Group (Laser): $p=0.0057$), and the interaction term at T=60 was also significant (Laser × T=60: $p=0.0151$), indicating a greater symptom reduction over time in the probiotic plus laser group compared with laser alone (Supplementary File 6). These findings support an improvement in dyspareunia over time in both groups, with more favorable estimates in the probiotic plus laser group; however, sexual activity frequency was not quantitatively recorded and should be considered when interpreting the magnitude of this symptom change.

Effect of treatments on introital pain

Both treatments were associated with a progressive reduction in introital pain severity over the study period, as assessed by Visual Analogue Scale (VAS) scores (Fig. 4). At baseline (T=0), mean introital pain scores were comparable between the two groups, with values of 8.28 in the laser-only group and 8.14 in the probiotic plus laser group (Supplementary File 7). Over time, a gradual decrease in introital pain was observed in both groups. At T=30, mean VAS scores decreased to 6.98 in the laser-only group and 6.57 in the probiotic plus laser group. Further reductions were observed at T=60 (5.66 and 4.86, respectively) and at T=90 (4.30 and 3.52, respectively) (Supplementary File 7). Post-hoc comparisons using Tukey's honestly significant difference test indicated significant reductions in pain scores over time within each group, while no statistically significant differences between groups were detected at any of the evaluated time points. Consistently, mixed-effects model analysis demonstrated a highly significant main effect of time on introital pain severity ($p<0.0001$), whereas neither the main effect of treatment group nor the group × time interaction reached statistical significance ($p=0.2703$ and $p=0.4892$, respectively), indicating a similar temporal pattern of symptom improvement in both treatment arms (Supplementary File 8). Parameter estimates from the linear mixed-effects model further supported these findings, showing significant time-related reductions in pain scores relative to T=90, with no evidence of a differential treatment effect between groups (Supplementary File 9). At T=90, the between-group mean difference in introital pain score was −0.77 VAS points (95% CI −2.10 to 0.55), corresponding to a small-to-moderate standardized effect size (Hedges' g=−0.36); however, the confidence interval was wide and compatible with no clear between-group difference.

Effect of treatments on vaginal dryness

Both treatments were associated with a progressive reduction in vaginal dryness severity over the study period, as assessed by Visual Analogue Scale (VAS) scores (Fig. 5). At baseline (T=0), mean vaginal dryness scores were

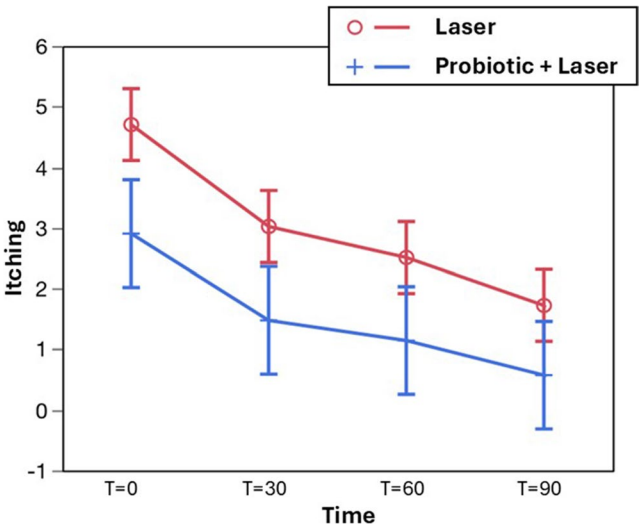

Fig. 5 Effect of treatments on vaginal dryness. Least squares mean Visual Analogue Scale (VAS) scores (0–10) are shown for the laser-only and probiotic plus laser groups at baseline (T=0) and at follow-up time points (T=30, T=60, and T=90 days). Both treatments were associated with a progressive reduction in vaginal dryness severity over time; however, the probiotic plus laser group exhibited significantly lower dryness scores from T=30 onward. Between-group differences and temporal patterns were supported by Tukey HSD post-hoc comparisons (Supplementary File 10) and linear mixed-effects model analyses (Supplementary Files 11 and 12)

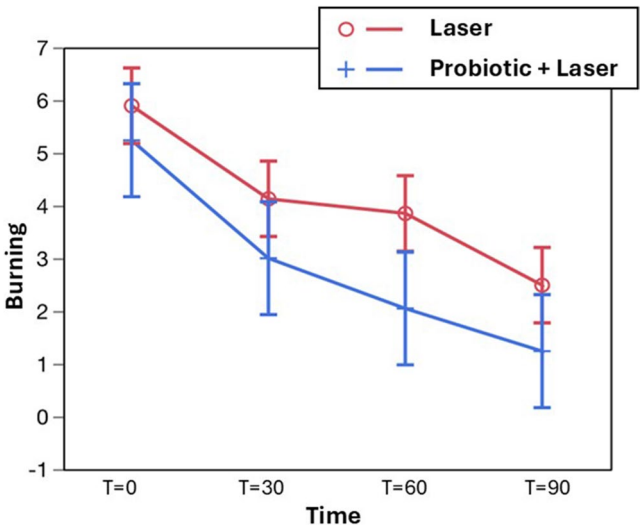

Fig. 6 Effect of treatments on vaginal itching. Least square mean Visual Analogue Scale (VAS) scores (0–10) are shown for the laser-only and probiotic plus laser groups at baseline (T=0) and at follow-up time points (T=30, T=60, and T=90 days). At baseline, vaginal itching scores differed significantly between groups, with higher values observed in the laser-only group. Both treatments were associated with a progressive reduction in itching severity over time, with consistently lower scores observed in the probiotic plus laser group during follow-up. Temporal patterns and between-group differences were supported by Tukey HSD post-hoc comparisons (Supplementary File 13) and linear mixed-effects model analyses (Supplementary Files 14 and 15)

comparable between groups, with values of 7.85 in the laser-only group and 7.62 in the probiotic plus laser group (Supplementary File 10). Over time, vaginal dryness scores decreased in both groups, with a more pronounced reduction observed in women treated with the combined probiotic and laser therapy. At T=30, mean VAS scores decreased to 6.79 in the laser-only group and 5.24 in the probiotic plus laser group. Further reductions were observed at T=60 (5.79 and 4.05, respectively) and at T=90 (4.32 and 3.10, respectively) (Supplementary File 10). Post-hoc comparisons using Tukey's honestly significant difference test demonstrated that vaginal dryness scores in the probiotic plus laser group were significantly lower than those observed in the laser-only group from T=30 onward, with the difference remaining evident at subsequent time points. Consistently, mixed-effects model analysis revealed a highly significant main effect of time ($p<0.0001$), a significant main effect of treatment group ($p=0.01525$), and a significant group × time interaction ($p=0.02062$), indicating a differential temporal response between the two interventions (Supplementary File 11). Parameter estimates from the linear mixed-effects model further supported these findings, showing a significant group effect at the reference time point (Group (Laser): $p=0.0206$) and a significant interaction at baseline (Laser × T=0: $p=0.0022$), consistent with a greater and earlier reduction in vaginal dryness severity in the probiotic plus laser group compared with laser therapy alone (Supplementary File 12). At T=90, the between-group mean difference in vaginal dryness score was −1.22 VAS points (95% CI −2.42 to −0.03), corresponding to a moderate standardized effect size (Hedges' g=−0.56), favoring the probiotic plus laser group.

Effect of treatments on vaginal itching

Both treatments were associated with a progressive reduction in vaginal itching severity over the study period, as assessed by Visual Analogue Scale (VAS) scores (Fig. 6). At baseline (T=0), vaginal itching scores were significantly different between the two groups, with higher mean values observed in the laser-only group (4.70) compared with the probiotic plus laser group (2.90), as indicated by Tukey HSD post-hoc comparisons (Supplementary File 13). Over time, vaginal itching scores decreased in both groups. In the laser-only group, mean VAS values declined to 3.02 at T=30, 2.51 at T=60, and 1.72 at T=90. In the probiotic plus laser group, a more pronounced reduction was observed, with mean scores of 1.48 at T=30, 1.14 at T=60, and 0.57 at T=90 (Supplementary File 13). Post-hoc analyses confirmed significant reductions in itching severity over time within each group, with consistently

lower scores observed in the probiotic plus laser group throughout follow-up. Mixed-effects model analysis corroborated these findings, showing a highly significant main effect of time ($p < 0.0001$) and a significant main effect of treatment group ($p = 0.00112$), while the group × time interaction was not statistically significant ($p = 0.66644$), indicating parallel temporal trends in symptom improvement between groups (Supplementary File 14). Parameter estimates from the linear mixed-effects model further supported the presence of an overall group effect at the reference time point (Group [Laser]: $p = 0.0011$) and significant time-related reductions in vaginal itching severity relative to T = 90 (Supplementary File 15).

Effect of treatments on vaginal burning

Both treatments were associated with a progressive reduction in vaginal burning severity over the study period, as assessed by Visual Analogue Scale (VAS) scores (Fig. 7). At baseline (T = 0), mean vaginal burning scores were comparable between the two groups, with values of 5.89 in the laser-only group and 5.24 in the probiotic plus laser group, with no statistically significant difference detected at baseline (Supplementary File 16). Over time, vaginal burning scores decreased in both groups. In the laser-only group, mean VAS values declined to 4.13 at T = 30, 3.85 at T = 60, and 2.49 at T = 90. In the probiotic plus laser group, a greater reduction was observed, with mean scores of 3.00 at T = 30, 2.05 at T = 60, and 1.24 at T = 90 (Supplementary File 16). Post-hoc comparisons using Tukey's honestly significant difference test confirmed significant reductions in burning severity over time within each group, with consistently lower scores observed in the probiotic plus laser group during follow-up. Mixed-effects model analysis supported these findings, demonstrating a highly significant main effect of time on vaginal burning severity ($p < 0.0001$) and a significant main effect of treatment group ($p = 0.02448$), while the group × time interaction did not reach statistical significance ($p = 0.33970$), indicating parallel temporal trends between the two treatment arms (Supplementary File 17). Parameter estimates from the linear mixed-effects model further confirmed the presence of a significant overall group effect at the reference time point (Group [Laser]: $p = 0.0245$) and significant time-related reductions in burning severity relative to T = 90 (Supplementary File 18). At T = 90, the between-group mean difference in vaginal burning score was −1.25 VAS points (95% CI −2.25 to −0.26), corresponding to a moderate standardized effect size (Hedges' g = −0.63), favoring the probiotic plus laser group.

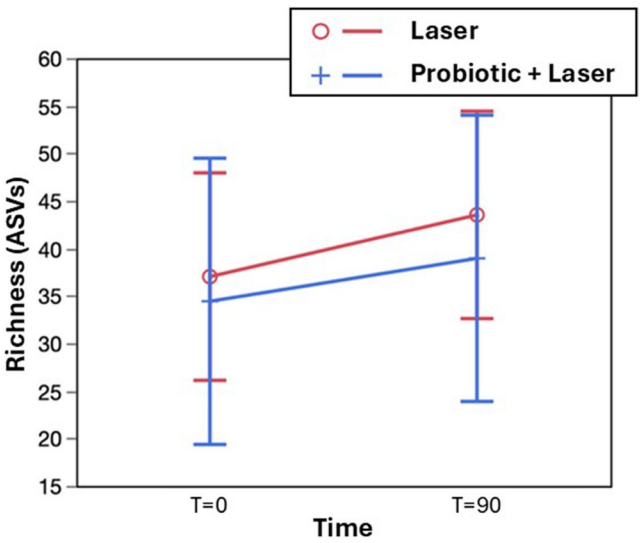

Fig. 7 Effect of treatments on vaginal burning. Least squares mean Visual Analogue Scale (VAS) scores (0–10) are shown for the laser-only and probiotic plus laser groups at baseline (T=0) and at follow-up time points (T=30, T=60, and T=90 days). Both treatments were associated with a progressive reduction in vaginal burning severity over time. Vaginal burning scores were consistently lower in the probiotic plus laser group during follow-up, with temporal trends and between-group differences supported by Tukey HSD post-hoc comparisons (Supplementary File 16) and linear mixed-effects model analyses (Supplementary Files 17 and 18)

Patients' global expectation and satisfaction with treatment

Patients' global expectation (PGE) and patients' global satisfaction (PGS) with treatment were assessed using a Likert-type scale ranging from 0 to 10 (Supplementary File 19). Mean PGE scores were comparable between the two study groups, with values of 8.02 in the control group and 7.81 in the treated group, and no statistically significant difference was observed between groups ($p > 0.05$). Similarly, mean PGS scores did not differ significantly between groups, with values of 7.98 in the control group and 8.00 in the treated group ($p > 0.05$). Both parametric and non-parametric statistical analyses consistently confirmed the absence of significant differences in expectation and satisfaction scores between treated and control participants. Overall, these findings indicate that patients' baseline expectations and perceived satisfaction with treatment were high and comparable between groups, and were not influenced by treatment allocation.

Safety and tolerability

Overall, the combined probiotic and laser treatment was well tolerated. No adverse events specifically attributable to oral administration of *L. crispatus* M247 were observed. All adverse events recorded during the study were mild

in severity (CTCAE grade 1), transient, self-limiting, and did not require any medical intervention. Reported events included headache, transient alterations in bowel habits, mild gastralgia, and anxiety or insomnia. The incidence of adverse events was comparable between treatment arms, with 12 events reported in the probiotic plus laser group and 26 events in the laser-only group. No serious adverse events were observed, and no participants discontinued the study due to treatment-related adverse effects.

Effect of treatments on vaginal microbiota richness

The effect of treatments on vaginal microbiota richness was evaluated by analyzing the number of observed amplicon sequence variants (ASVs) in the two study groups at baseline (T = 0) and at the end of follow-up (T = 90) (Fig. 8). At T = 0, mean ASV richness values were comparable between groups, with mean values of 37.03 in the control group and 34.43 in the treated group. At T = 90, a modest increase in ASV richness was observed in both groups, with mean values of 43.53 in controls and 38.95 in treated subjects. As shown in Fig. 8, both groups exhibited a similar temporal pattern, characterized by a slight increase in mean ASV richness from T = 0 to T = 90. However, substantial inter-individual variability was evident at both time points, as illustrated by the distribution of individual ASV values (Supplementary File 20). Mixed-design (split-plot) analysis of variance revealed no statistically significant main effect of time ($p = 0.394$), indicating the absence of a significant change in bacterial richness over the study period. Likewise, no significant main effect of treatment group was detected ($p = 0.603$), and the group × time interaction was not statistically significant ($p = 0.878$), indicating that the temporal evolution of ASV richness was comparable between treated and control women. Descriptive statistics further supported these findings, showing overlapping measures of central tendency and dispersion between groups at both time points (Supplementary File 21). Overall, vaginal microbiota richness, as measured by ASV counts, remained stable over time and was not significantly influenced by treatment allocation, despite a modest, non-significant increase in mean values observed in both groups. Overall, these results indicate that vaginal microbiota richness, as measured by ASV counts, remained stable over the study period and was not significantly influenced by treatment allocation, despite a modest, non-significant increase in mean values observed in both groups.

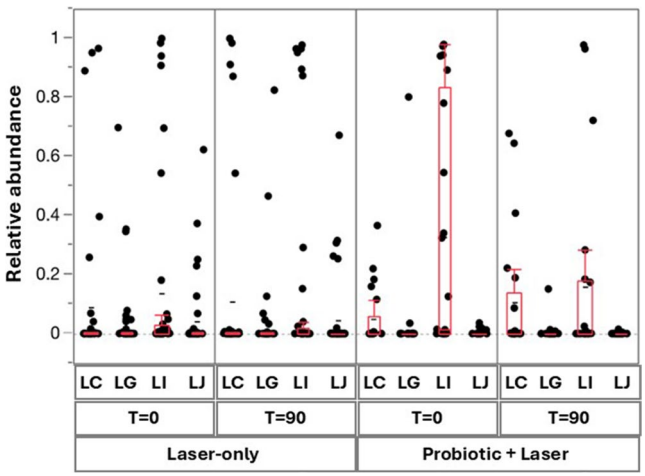

Fig. 8 Effect of treatments on vaginal microbiota richness (ASVs) over time. Least squares mean values of vaginal microbiota richness, expressed as the number of observed amplicon sequence variants (ASVs), are shown for the control and treated groups at baseline (T=0) and at follow-up (T=90). Both groups exhibited a slight increase in mean ASV richness over time; however, variability was high and no statistically significant effects of time, treatment group, or their interaction were detected. The distribution of individual ASV values and descriptive statistics are reported in Supplementary Files 20 and 21

Effect of treatments on vaginal community state types (CSTs)

Vaginal microbiota composition was further evaluated by classifying samples into Community State Types (CSTs) based on the relative abundance of Lactobacillus spp. At each time point, samples with a Lactobacillus relative abundance < 50% were classified as CST IV (non–Lactobacillus-dominant), whereas samples with Lactobacillus ≥ 50% were classified as CST I, II, III, or V according to the dominant *Lactobacillus* species (*L. crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii*, respectively). The distribution of CSTs by treatment group and time point is reported in Table 2. At baseline (T = 0), CST IV was the most prevalent community type in both groups, accounting for 70.0% of samples in the laser-only group and 57.1% in the probiotic plus laser group. Among Lactobacillus-dominant communities, CST III (*L. iners*–dominated) was the most frequent subtype at baseline, particularly in the probiotic plus laser group (38.1%). At follow-up (T = 90), CST IV remained the most common community type in both treatment arms, with only modest fluctuations over time. When CSTs were analyzed individually, a divergent pattern emerged in the probiotic plus laser group that was not observed in the laser-only group. In treated women, the proportion of CST I (*L. crispatus*–dominated communities) increased from 0.0% at baseline to 14.3% at follow-up, while CST III (*L. iners*–dominated communities) showed a concomitant decrease from 38.1% to 14.3%. In contrast, CST II and CST V remained rare at both time points, and the laser-only group showed only minimal changes in CST distribution, with CST I increasing slightly from 10.0% to 12.5% and CST III remaining stable

Table 2 Community State Type (CST) distribution by group and time

Group	Time	CST I	CST II	CST III	CST IV	CST V
Laser-only	T=0	4 (10.0%)	1 (2.5%)	6 (15%)	28 (70%)	1 (2.5%)
Laser-only	T=90	5 (12.5%)	1 (2.5%)	6 (15%)	27 (67.5%)	1 (2.5%)
Probiotic + laser	T=0	0 (0%)	1 (4.8%)	8 (38.1%)	12 (57.1%)	0 (0%)
Probiotic + laser	T=90	3 (14.3%)	0 (0%)	3 (14.3%)	15 (71.4%)	0 (0%)

CST Community State Type

at 15.0%. Although formal statistical testing did not identify significant differences due to the limited sample size and the categorical nature of CST data, the opposite direction of change observed for CST I and CST III in the probiotic plus laser group may suggest a biologically relevant trend toward a more favorable, L. crispatus–dominated vaginal microbiota following combined treatment; however, these CST findings should be regarded as exploratory/hypothesis-generating rather than confirmatory.

Effect of treatments on vaginal *Lactobacillus* species distribution

The distribution of the main vaginal Lactobacillus species was further evaluated by analyzing their relative abundances using non-parametric descriptors, focusing on interquartile ranges (IQRs) to account for the highly skewed distribution of microbiota data. The variability of *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* across treatment groups and time points is illustrated in Fig. 9, while quantitative summaries based on IQRs are reported in Supplementary File 22. At baseline (T=0), both groups showed wide inter-individual variability, particularly for *L. iners*. In the probiotic plus laser group, *L. iners* displayed a markedly broad IQR (83.64%), indicating a heterogeneous distribution with a subset of women harboring high relative abundances of this species. In contrast, the laser-only group exhibited a narrower IQR for *L. iners* (2.76%), suggesting lower dispersion and generally limited representation of this taxon. At the same time point, *L. crispatus* showed limited variability in the laser-only group (IQR 0.36%), whereas a wider IQR was observed in the probiotic plus laser group (IQR 6.06%), indicating the presence of individuals with higher *L. crispatus* abundance. At follow-up (T=90), distinct patterns emerged between treatment arms. In the probiotic plus laser group, the IQR of *L. crispatus* increased substantially to 13.78%, reflecting an expansion in the upper distribution of this species and suggesting a shift toward *L. crispatus* enrichment in a subset of treated women. Concurrently, the IQR of *L. iners* decreased markedly to 17.90%, indicating reduced dispersion and a contraction of high-abundance *L. iners* profiles. In contrast, the laser-only group showed relatively stable and narrow IQRs for both *L. crispatus* (0.28%) and *L. iners* (2.03%) between T=0 and T=90, consistent

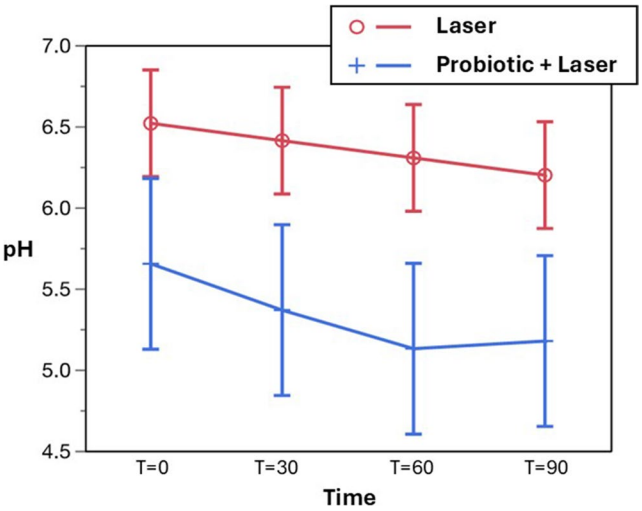

Fig. 9 Distribution of selected *Lactobacillus* species over time in the two treatment groups. Box-and-whisker plots showing the distribution of relative abundances of *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* at baseline (T=0) and follow-up (T=90) in the probiotic plus laser (left panel) and laser-only (right panel) groups. Boxes represent interquartile ranges (IQRs), horizontal lines indicate medians, and whiskers denote the distribution of values outside the IQR. Individual points represent single subjects. The figure highlights differences in inter-individual variability and temporal trends in *Lactobacillus* species distribution between treatment arms. LC: *L. crispatus*; LG: *L. gasseri*; LI: *L. iners*; LJ: *L. jensenii*

with minimal compositional modulation over time. Inferential statistical analyses supported these descriptive findings. For *L. crispatus*, within-subject comparisons performed using the Wilcoxon signed-rank test demonstrated a significant increase from T=0 to T=90 in the probiotic plus laser group ($p=0.0474$), whereas no significant temporal variation was observed in the laser-only group ($p=0.7608$). Between-group comparisons conducted using the Mann–Whitney U test showed overlapping distributions at both baseline (T=0; $p=1.000$) and follow-up (T=90; $p=0.275$). For *L. gasseri*, Wilcoxon signed-rank testing revealed no significant within-group changes over time in either the probiotic plus laser group ($p=0.5164$) or the laser-only group ($p=0.4729$), and no significant differences between groups were detected at T=0 ($p=0.490$) or T=90 ($p=0.219$). Analysis of *L. iners* showed a significant reduction from T=0 to T=90 in the probiotic plus laser group (Wilcoxon

signed-rank test, $p=0.0486$), while no significant temporal change was observed in the laser-only group ($p=0.9527$). Between-group comparisons revealed a significant difference at baseline (T=0; $p=0.032$), indicating non-overlapping starting distributions between treatment arms; this baseline imbalance may have partially influenced the magnitude of longitudinal changes. However, no significant difference between groups was detected at follow-up (T=90; $p=0.552$), suggesting convergence of *L. iners* abundance over time. Finally, for *L. jensenii*, Wilcoxon signed-rank analysis showed no significant temporal variation in either the probiotic plus laser group ($p=0.5031$) or the laser-only group ($p=0.6506$), and Mann–Whitney U testing demonstrated no significant differences between groups at baseline (T=0; $p=0.706$) or follow-up (T=90; $p=0.552$). Overall, the combined descriptive and inferential analyses indicate that the probiotic plus laser treatment was associated with a redistribution of *Lactobacillus* species characterized by an increase in *L. crispatus* and a concomitant reduction in *L. iners*, whereas *L. gasseri* and *L. jensenii* remained stable. Despite the presence of a baseline imbalance in *L. iners*, the observed convergence at follow-up and the parallel expansion of *L. crispatus* suggest a biologically meaningful modulation toward a more favorable vaginal Lactobacillus profile following combined treatment.

Additional vaginal *Lactobacillus* and non-*Lactobacillus* species

In addition to the characterization of the four dominant *Lactobacillus* species defining CSTs, the presence of other vaginal *Lactobacillus* species was evaluated by analyzing their mean relative abundances across groups and time points. These included *L. animalis*, *L. salivarius*, *L. paracasei*, *L. rhamnosus*, *L. backii*, *L. heilongjiangensis*, *L. delbrueckii*, *L. oris*, *L. plantarum*, *L. fermentum*, *L. agilis*, *L. aviarius*, *L. acidophilus*, *L. reuteri*, *L. mucosae*, *L. amylophilus*, and unidentified *Lactobacillus* spp. (Supplementary Files 22 and 23). In both the laser-only group and the probiotic plus laser group, the mean relative abundances of these species at T=0 and T=90 were consistently very low, with values close to zero for most taxa, indicating a marginal contribution of these species to the overall vaginal microbiota. Longitudinal and between-group comparisons were performed using mixed-design analysis of variance or mixed-effects models when distributional assumptions were met, while non-parametric tests were applied for taxa showing non-normal distributions or high inter-individual variability. These analyses did not reveal significant effects of time, group, or time × group interaction for any of the additional *Lactobacillus* species ($p>0.05$ for all comparisons), supporting the conclusion that the observed microbiota modulation was primarily driven by changes in dominant *Lactobacillus* taxa rather than by shifts in minor or infrequently detected species. Descriptive statistics for non-*Lactobacillus* taxa in the laser-only and probiotic plus laser groups are provided in Supplementary Files 24 and 25, respectively.

Discussion

Vaginal CO_2 laser therapy has become a widely used therapeutic option for the management of vulvovaginal atrophy (VVA) in postmenopausal women, particularly in those who are unwilling or unable to use local estrogen therapy. The majority of published clinical studies describe laser treatment as safe as effective in improving vaginal symptoms, including dryness, dyspareunia, and burning, as well as objective parameters such as vaginal pH and epithelial trophism [31–33]. These findings have supported the diffusion of this approach in routine clinical practice and its inclusion among non-hormonal therapeutic strategies for VVA. However, in recent years, the efficacy of vaginal laser therapy has been questioned by several controlled trials and systematic evaluations, which have reported limited or no superiority of laser treatment compared with sham procedures or standard care [12, 34, 35]. These reports have highlighted the need for further controlled studies to better define the real clinical benefit of laser therapy, the magnitude of its effects, and the patient populations most likely to respond. In this context, we deliberately designed the present study to reassess the clinical efficacy of CO_2 laser therapy, assigning a numerical advantage to the laser-only group through a predefined 2:1 randomization ratio, in order to obtain robust estimates of laser-related effects. Although randomization was applied, some baseline differences between groups were observed, including age, smoking status, bowel discomfort, and mood swings or insomnia. These imbalances are likely attributable to the relatively limited sample size and the predefined 2:1 allocation ratio. Importantly, all of these baseline characteristics were more prevalent or more pronounced in the probiotic plus laser group. As older age, smoking, gastrointestinal discomfort, and sleep or mood disturbances are commonly associated with greater symptom burden and potentially reduced treatment responsiveness, such imbalances would be expected to bias the results toward underestimating, rather than exaggerating, any additional benefit of adjunctive probiotic therapy. Furthermore, the longitudinal mixed-effects modeling approach, based on within-subject changes over time and on group × time interactions, reduces the influence of baseline heterogeneity on treatment effect estimation. In addition, post hoc covariate-adjusted sensitivity analyses including age, current smoking status, and baseline endpoint values yielded estimates that were directionally

consistent with the main findings for pH and key clinical outcomes. Nevertheless, residual confounding cannot be entirely excluded and should be addressed in future larger studies using stratified randomization and prespecified covariate-adjusted models. Beyond symptom relief, increasing attention has been directed toward the role of the vaginal microbiota in vaginal health. A *Lactobacillus*-dominated microbiota is widely considered eubiotic, and several studies have shown that a community state type (CST) characterized by dominance of *L. crispatus* and low microbial richness is associated with vaginal homeostasis, reduced inflammation, and lower susceptibility to infections [36–40]. This concept has been extensively validated in reproductive-age women and is increasingly recognized as relevant also in postmenopausal women, despite the profound hormonal changes associated with menopause [41–44]. Among lactobacilli, *L. crispatus*–based probiotics remain relatively underutilized compared with other *Lactobacillus* species. To date, mainly two *L. crispatus* strains have been evaluated in interventional studies: the CTV-05 strain, administered vaginally, and the M247 strain, administered orally [45–50]. The latter has been shown to reach the vaginal ecosystem after oral intake, promoting vaginal colonization and favoring a shift toward CST I in premenopausal populations [9, 51]. Nevertheless, no data were previously available regarding the use of *L. crispatus* M247 in postmenopausal women, in whom the low-estrogen environment could theoretically limit or prevent effective colonization. On this basis, we designed a controlled study to evaluate the add-on effect of oral *L. crispatus* M247 in women undergoing vaginal CO_2 laser therapy. Our results indicate that laser therapy alone was associated with clinical improvement over time in vaginal pH and VVA-related symptoms. However, laser treatment by itself did not induce detectable changes in vaginal microbiota composition when analyzed at species-level resolution using 16 S rRNA gene sequencing. This finding contrasts with some previous reports suggesting an increase in lactobacilli following laser therapy, possibly reflecting differences in analytical depth or taxonomic resolution, as most previous studies relied on genus-level or non-NGS methodologies. Importantly, the addition of L. crispatus M247 to laser therapy was associated with more favorable estimates for several clinical outcomes at T90; however, the corresponding confidence intervals were relatively wide for some endpoints, indicating limited precision in this sample. Although no universally accepted minimal clinically important difference is available for each individual GSM symptom assessed by symptom-specific VAS, prior sham-controlled laser literature has considered a 50% reduction in symptom severity on VAS as clinically meaningful [52]. Interpreted in this context, the magnitude of improvement observed for selected symptom domains in our study may be clinically relevant, while still requiring cautious interpretation in light of the sample size, confidence interval width, and open-label design. Because the study was open-label and several clinical outcomes were self-reported, these symptom improvements should also be interpreted in light of possible expectation-related effects. Vaginal dryness was assessed using a pragmatic symptom-specific VAS, which allowed simple and repeated longitudinal assessment and is consistent with approaches adopted in previous GSM laser studies [53]. However, a formally validated GSM/VVA-specific patient-reported outcome instrument, such as the Vulvovaginal Symptoms Questionnaire (VSQ) or the Day-to-Day Impact of Vaginal Aging (DIVA) questionnaire, was not used, and this should be considered when interpreting construct validity. This also applied to dyspareunia, which was clinically relevant in this cohort because all enrolled women were sexually active, or attempting vaginal intercourse, during the study period. Nevertheless, the frequency of sexual activity was not quantitatively recorded and this should be considered when interpreting the magnitude of dyspareunia improvement. For vaginal pH, the probiotic plus laser group showed consistently lower values during follow-up, but the longitudinal analysis did not demonstrate a significantly different temporal response compared with laser alone. However, the microbiota-related findings, particularly the CST-level changes, should be interpreted cautiously because of the limited sample size for microbiota analyses and the absence of formal statistical significance for CST shifts. These observations are therefore best considered hypothesis-generating. These findings suggest that probiotic supplementation may enhance the clinical benefits of laser therapy while simultaneously promoting a vaginal microbial environment more conducive to long-term vaginal health.

This study has several limitations that should be acknowledged. First, although participants were assigned according to a pre-generated randomization sequence, the study adopted a 2:1 allocation ratio without formal blocking or stratification, and no additional allocation concealment procedure was implemented. In the context of the modest sample size, these design features may have contributed to the baseline differences observed in some demographic and clinical variables, such as age, smoking habits, mood swings, and bowel discomfort. These imbalances may have acted as confounders and should therefore be taken into account when interpreting the internal validity of the study and the between-group comparisons. Although post hoc covariate-adjusted sensitivity analyses including age and current smoking status yielded estimates consistent with the main findings, residual confounding from other baseline differences cannot be fully excluded. Notably, several baseline characteristics were less favorable in the probiotic group, which would be expected to bias any treatment effect toward the null rather than to overestimate it. Regarding

vaginal pH, although baseline values were numerically different, the baseline Tukey comparison was not statistically significant and the group × time interaction was not statistically significant. Therefore, the pH results should be interpreted as reflecting a stable between-group separation rather than definitive evidence of a treatment-specific temporal effect. In addition, the modest sample size resulted in relatively wide confidence intervals for several clinical effect estimates, limiting precision and making small-to-moderate between-group differences difficult to estimate reliably. Second, the control group received laser therapy alone, whereas the addition of an active placebo—such as a non-vaginally targeted probiotic—might have helped control for non-specific effects related to oral supplementation. This limits the ability to isolate the specific contribution of *L. crispatus* M247. Third, no correction for multiple testing was applied to secondary outcomes. As a result, p-values should be interpreted as exploratory, and emphasis should be placed on overall consistency across endpoints rather than isolated significance levels. Fourth, microbiota analyses were limited by the absence of repeated intermediate time points between baseline and day 90. As such, potential short-term changes or fluctuations in community state types (CSTs) may have been missed. In addition, the relatively small sample size available for microbiota profiling, particularly for CST-based categorical analyses, limits statistical power and requires these findings to be interpreted as exploratory/hypothesis-generating. Fifth, this was an open-label trial, and although participants completed symptom scales themselves, reporting bias cannot be fully excluded. In particular, because several key clinical outcomes were based on self-reported VAS measures, expectation and placebo effects may have contributed to the observed symptom changes. Although questionnaires were completed at home using the same written format and fixed anchors and were returned without possibility of alteration, no formal blinding or scripted counseling procedure was implemented. In addition, the absence of a sham-laser control arm limits the extent to which symptom improvements can be distinguished from non-specific procedural and expectation-related effects. Vaginal dryness was assessed using a pragmatic symptom-specific VAS rather than a formally validated GSM/VVA-specific patient-reported outcome instrument. Although this approach facilitated repeated symptom monitoring, it may limit construct validity compared with disease-specific validated questionnaires. Likewise, although dyspareunia was assessed in a cohort of women who were all sexually active, or attempting vaginal intercourse, during the study period, the frequency of sexual activity was not quantitatively recorded as a longitudinal variable. This may limit the precision with which changes in dyspareunia severity can be interpreted over time. Finally, the follow-up duration was limited to 90 days, so the durability of the clinical and microbiological improvements remains to be established in longer-term studies.

Despite these limitations, the present study introduces several novel aspects that distinguish it from previous investigations in this field. To our knowledge, this is the first study to evaluate changes in the vaginal *Lactobacillus* population at species-level resolution in postmenopausal women undergoing vaginal CO_2 laser therapy, by combining 16 S rRNA gene sequencing with internal transcribed spacer (ITS) analysis. Previous studies assessing the interaction between laser therapy and the vaginal microbiota have largely relied on genus-level analyses or non–next-generation sequencing approaches, such as microscopy, culture-based methods, or Nugent scoring, which do not allow precise characterization of species-specific dynamics [54–58]. Moreover, this study is the first to investigate, in a controlled setting, the combined effects of vaginal laser therapy and adjunctive probiotic supplementation with a *L. crispatus* strain on both clinical outcomes and vaginal microbiota composition. To date, no clinical studies have simultaneously evaluated symptom improvement and microbiota modulation following probiotic add-on therapy in postmenopausal women with VVA. The integration of high-resolution microbiota profiling with clinical endpoints therefore provides a more comprehensive assessment of therapeutic effects and offers new insight into the potential role of targeted probiotic strategies in this population. Taken together, these findings suggest that combining tissue-targeted and microbiota-targeted interventions may represent a complementary and biologically coherent strategy for the management of vulvovaginal atrophy in postmenopausal women.

Conclusions

Despite its preliminary nature and inherent limitations, this study suggests that vaginal CO_2 laser therapy was associated with clinical improvement in postmenopausal women with VVA and provides preliminary evidence that adjunctive oral administration of L. crispatus M247 may be associated with additional clinical and microbiological benefits.

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Data availability All data analyzed during this study are included in this article and/or in Supplementary Files. The data that support the gut microbiota findings of this study were generated by a third-party service provider. Due to contractual and proprietary restrictions, the raw sequencing reads cannot be deposited in a public repository and are not directly available from the authors. Anonymized individual-level microbiota analysis reports, including relative abundances of bacterial *taxa* used for all statistical analyses in this study, are available from the corresponding author upon reasonable request. All data are fully anonymized and contain no information that could identify individual participants.

Declarations

Ethics approval and consent to participate The study protocol was reviewed and approved by the Ethics Committee of the University of Urbino "Carlo Bo" (Italy) (Minutes of Session No. 93, April 24, 2025). This study was registered at ClinicalTrials.gov (Identifier: NCT06978907). Written informed consent to participate was obtained from all individual participants included in the study.

Inclusion of identifiable human data No potentially identifiable images or data are presented in this study.

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