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ORIGINAL ARTICLE

Oral administration of *Lactobacillus crispatus* M247 to papillomavirus-infected women: results of a preliminary, uncontrolled, open trial

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ABSTRACT

BACKGROUND: Vaginal microbiotas can be clustered into five different possible categories (CST I to V), according to their bacterial dominance. In CST I, the dominance of *Lactobacillus crispatus* seems to correlate with better vaginal health and with a lower incidence of *sine causa* infertility, preterm delivery, bacterial vaginosis, and viral (including human papillomavirus; HPV) infection. According to the same method of classifying the vaginal microbiome, CST IV (non-*Lactobacillus*-dominated) demonstrates a higher incidence of disorders.
METHODS: In an open, non-controlled study, we enrolled 35 HPV-positive women who mostly (N.=24) demonstrated CST IV status, with the other individuals categorized as having either CST III (N.=10) or CST II (N.=1) microbiotas.
RESULTS: After 90 days of oral treatment with a probiotic (*L. crispatus* M247) we observed a reduction of approximately 70% in HPV positivity and a significant change in CST status with 94% of women now classified as CST I.
CONCLUSIONS: Despite the limitations of our study, it is the first demonstration that it is possible to intervene orally with an *L. crispatus* probiotic to bring about a change in CST status and, in parallel, increased HPV clearance.

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KEY WORDS: Papillomaviridae; Microbiota; Uterine cervical neoplasms.

Since 2011,[1] the scientific community has been accepting the concept of Community State Type (CST), where vaginal microbiotas are clustered into five different possible categories, according to their bacterial dominance. When the vaginal microbiota is primarily dominated by one of the four most common vaginal *Lactobacillus* species, that is, *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, the CST is numbered I, II, III, and V, respectively. When the vaginal microbiota is dominated by a bacterial species other than lactobacilli, such as *Prevotella*, *Gardnerella*, *Atopobium*, *Streptococcus*, and *Sneathia*, the cluster is called CST IV. Some authors in the field, although not all, separate the CST IV cluster into sub-clusters named A and B, where CST IV A appears to be dominated by *Anaerococcus*, *Corynebacterium*, *Finegoldia*, and *Streptococcus* along with *L. iners* or a small percentage of other *Lactobacillus* species, while for CST

IV B dominant bacteria seem to be *Prevotella*, *Gardnerella*, *Atopobium*, *Mobiluncus*, *Parvimonas*, *Peptoniphilus*, and other bacteria.[2] The five CSTs can be distinguished also by richness, with the *Lactobacillus*-dominated CSTs being less biodiverse and CST IV A being less biodiverse than CST IV B.[3] A number of studies suggest that a *Lactobacillus*-dominated vaginal microbiota, and in particular an *L. crispatus*-dominated CST, could play an essential role in women's health, positively affecting infertility, bacterial vaginosis, sexually transmitted diseases, pelvic inflammatory disease, and adverse obstetric outcomes.[4-8] The relationship between CST I and women's health, besides being a statistically significant correlation, could also be causative as the results obtained in the first preliminary trial of vaginal microbiota transplantation would seem to demonstrate.[9] Cross-sectional and longitudinal studies have shown that the *L. crispatus*-dominated CST is observed more frequently in women without human papillomavirus (HPV) infection and cancer lesions, whereas CST III and CST IV are more common in HPV-infected women and in patients with cancer lesions.[10-13] HPV infection is among the most common sexually transmitted infections in the world, with the highest prevalence among young women.[14, 15] It is the main cause of cervical cancer and many other cancer types.[16] The synergistic effects of persistent high-risk HPV infection, changes in the cervical microenvironment, and other auxiliary carcinogenic factors (*i.e.*, prolificacy, sexual disorders, and smoking) facilitate the development of cervical precancerous lesions. HPV types can be divided into non-oncogenic HPVs, mainly found in condyloma, and oncogenic HPVs, as those observed in cancer cases.[17] The most common and most investigated HPV types in cervical cancer are HPV16 and HPV18. These are responsible for approximately 70% of cervical cancer cases worldwide.[18, 19] In Western countries, all girls between the ages of 10 and 12 years are offered free vaccination with the quadrivalent Gardasil vaccine against HPV serotypes 6, 11, 16, and 18. Despite the introduction of Gardasil 9, covering strains 6, 11, 16, 18, 31, 33, 45, 52, and 58, the total HPV infection prevalence caused by HPV types that are not covered by the vaccine is still high, indicating that more interventions to reduce these HPV infections are still needed. Despite the significant association between CST *L. crispatus* dominance and vaginal health, and the availability of strains of *L. crispatus*, including *L. crispatus* CTV-05 that was recently successfully clinically tested by vaginal administration to women affected by recurrent bacterial vaginosis,[20] the oral use of *L. crispatus* strains to modify the CST and/or to increase HPV clearance in HPV-positive women has never been previously attempted. *L. crispatus* M247 is a well-documented probiotic that demonstrates fecal and vaginal colonizing properties after oral administration.[21-29] We have therefore used this strain in a preliminary, longitudinal, uncontrolled, and open study enrolling HPV-positive women, to assess its possible impact both in terms of the CST and on HPV status. The aim of the study was to evaluate a possible correlation between the vaginal microbiota and the 90-day persistence of HPV infection - both high-risk (HR) and low-risk (LR) strains - in the cervical-vaginal area. Our results seem to demonstrate, in addition to confirming the safety profile of the treatment, the existence of a relationship between (1) the oral administration of the *L. crispatus* strain, (2) the increased number of women with CST I classification after its use, and (3) apparently increased HPV clearance.

Materials and methods

Study type and aim, enrolled subjects, inclusion and exclusion criteria

Our observational, open, pilot study, with no power analysis performed in advance, was conducted using a group of 35 women with HPV cervical infection, with normal or abnormal cervical cytology, and recurrent vaginal infections. Both the vaginal microbiota and the HPV status were analyzed at enrollment and after 90 days of oral treatment with *L. crispatus* M247. The initial study group included 40 sexually active women, aged between 18 and 65 years old, who routinely attended the Colposcopy and Cervical-Vaginal Pathology Clinic and Vulvar at the Department of Gynecology and Obstetrics of the Tor Vergata University (Rome, Italy) between July 2019

and May 2020 for the prevention, diagnosis, and treatment of HPV-related genital disease. After giving a detailed explanation of the aims of the study, 35 women agreed to take part in the trial. They provided informed consent to publish the results and completed all the necessary paperwork relating to privacy. All patient data were completely anonymized, and the study was performed in accordance with the ethical standards established by the Tor Vergata University institutional authorities and with the approval of the Ethical Committee (Authorization number: 218/20). The inclusion criteria were being a sexually active woman aged between 18 and 65, with a positive HPV DNA test (also if smear-negative), and with a Pap (Papanicolaou) test reporting low-grade cervical cytology as atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesion (L-SIL) and negative for intraepithelial or malignant lesions (women not revealing HPV-correlated lesions on the cervix, both low grade G1 and high grade G2) or for reactive cellular modifications associated with inflammation and/or tissue atrophy. We therefore excluded women who had never had vaginal sexual intercourse, or those who were pregnant or breastfeeding; women with a negative history of HPV-related cervicovaginal infections; women with cervical cytology indicating for high-grade squamous intraepithelial lesion (H-SIL); women with previous cervical intraepithelial neoplasia (CIN) 2 or CIN 3 and/or squamous cell carcinoma of the uterine cervix; women who had received HPV vaccination.

Pap test, HPV DNA test, HPV RNA test

The Pap test was performed by collecting eso- and endocervical cells using Ayre's spatula and a cytobrush, respectively. The cells were streaked on a glass slide, spray-fixed, and sent to the pathology laboratory for cytological diagnosis. Smears were classified according to the 2001 Bethesda System: normal; ASCUS, atypical squamous cells high-grade lesion (ASCH), L-SIL; and H-SIL.[30] The HPV DNA test was performed using the Anyplex™II HPV28 assay (Seegene, Seoul, South Korea). Total DNA/RNA was extracted using the NucliSENS® easyMAG® kit on the eMAG™ automatic extractor (bioMérieux SA, F-69280 Marcy l'Étoile, France). The Anyplex™II HPV28 assay simultaneously detects 28 HPV types including 19 HR-HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 LR-HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70).[31] E6/E7 mRNA for the oncogenic HPV types 16, 18, 31, 33, and 45 was detected using the NucliSENS EasyQ® HPV kit (bioMérieux SA, F-69280 Marcy l'Étoile, France) following the manufacturer's instructions.[32]

Sample collection, bacterial DNA extraction, *16S rRNA* gene and ITS PCR amplification and sequencing

For the purposes of this study, a total of 35 vaginal swab samples were collected in sterile tubes containing 1 mL of DNA/RNA Shield from Zymo Research and stored until bacterial DNA extraction. Vaginal samples were subjected to DNA extraction using the ZymoBIOMICS DNA Miniprep Kit following the manufacturer's instructions (Zymo Research, Irvine, CA, USA). Partial *16S rRNA* gene sequences were amplified from extracted DNA using the primer pair Probio_Uni/Probio_Rev, targeting the V3 region of the *16S rRNA* gene sequence.[33] Illumina adapter overhang nucleotide sequences were added to the partial *16S rRNA* gene-specific amplicons, which were further processed employing the 16S 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 *16S 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.[34] 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, *16S rRNA* operational taxonomic units (OTUs) were defined at 100% sequence homology using DADA2;[35] 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.[36] All reads were classified to the lowest possible taxonomic rank using QIIME2[37, 38] and a reference dataset from the SILVA database.[39] Biodiversity within a given sample (α-diversity) was calculated using the Chao1 and Shannon indexes. The range of similarities were calculated between values 0 and 1. PCoA representations of beta-diversity were obtained using QIIME2.[37, 38] Regarding bacterial DNA extraction, *Lactobacillus* ITS PCR amplification, and sequencing, vaginal samples were subjected to DNA extraction using the ZymoBIOMICS DNA Miniprep Kit following the manufacturer's instructions (Zymo Research). 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 *16S rRNA* and the 23S rRNA genes within the rRNA locus.[40] Illumina adapter overhang nucleotide sequences were added to the partial ITS amplicons, which were further processed employing the 16S 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 *16S rRNA* microbial profiling analyses. Following sequencing, the .fastq files were processed using a custom script based on the QIIME software suite.[35] 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.[38] All reads were classified to the lowest possible taxonomic rank using QIIME2[37, 38] and a reference dataset, consisting of an updated version of the *Lactobacillus* ITS database.[40]

CST classification

According to most of the published data regarding the classification of a particular vaginal CST, we have established a definition of a *Lactobacillus*-dominated CST as when lactobacilli constitute ≥80% of the total sample and to define the exact type of dominant *Lactobacillus* spp. when there is ≥80% dominance among the mix of different lactobacilli.[1-5]

Tested product

The probiotic product used in our clinical study corresponds to Crispact® sachets. Each sachet contains no fewer than 20 billion colony forming units (CFU) of *L. crispatus* M247 (LMG-P-23257).[21] The product is manufactured by Labomar SpA (Istrana, Treviso, Italy) and traded by Pharmextracta SpA (Pontenure, Piacenza, Italy). The product was notified to the Italian Health Authorities in 2019 (March 1st) with the notification number 115450.

Statistical analysis

All recorded data were initially entered into an Excel database (Microsoft, Redmond, Washington, USA). Statistical analysis was carried out using version 15.0 of the Statistical Package for the Social Sciences Windows software (SPSS, Chicago, IL, USA). To evaluate the significance of the association between features (for example, smoking, use of contraceptives) with HPV and/or CST status, Fisher analysis was used (Table I). The relationship between CST status and HPV DNA evolution within the same subjects, before and after treatment, was assessed using the Wil-

TABLE I.—*Features of the enrolled women before (T=0) and after 3 months of oral treatment (T=90) with* L. crispatus *M247.*

	T=0								T=90	
Patient	Smoker	Pill User	Symptoms of Vaginosis	Pap Test	Colposcopy	HPV, RNA	HPV, DNA	CST	HPV, DNA	CST
1	Yes	Yes	Yes	ASCUS	Neg	Neg	HR+	IV	Neg	I
2	Yes	Yes	Yes	L-SIL	Neg	Neg	HR+	IV	Neg	I
3	Yes	No	No	L-SIL	Neg	Neg	LR+	III	Neg	I
4	Yes	Yes	No	Neg	Neg	Neg	HR+	IV	Neg	I
5	Yes	Yes	Yes	ASCUS	Neg	Neg	HR+	IV	Neg	I
6	Yes	Yes	Yes	ASCUS	Neg	Neg	HR+	IV	Neg	I
7	Yes	Yes	No	Neg	Pos	Neg	HR+	IV	Neg	I
8	Yes	Yes	No	Neg	Neg	Neg	HR+	IV	Neg	I
9	Yes	Yes	No	ASCUS	Neg	Neg	HR+	IV	Neg	I
10	No	Yes	No	Neg	Neg	Neg	HR+	III	Neg	I
11	No	No	No	L-SIL	Neg	Neg	HR+	III	Neg	I
12	No	Yes	No	L-SIL	Neg	Neg	LR+	III	Neg	I
13	Yes	Yes	No	Neg	Pos	Neg	HR+	IV	HR+	III
14	Yes	Yes	Yes	Neg	Neg	Neg	HR+	IV	Neg	I
15	Yes	Yes	No	Neg	Pos	Neg	HR+	IV	HR+	I
16	No	No	No	Neg	Neg	Neg	LR+	III	Neg	I
17	Yes	Yes	Yes	Neg	Neg	Pos	HR+	IV	HR+	I
18	Yes	Yes	Yes	ASCUS	Neg	Neg	HR+	IV	Neg	I
19	Yes	Yes	Yes	ASCUS	Neg	Neg	HR+	IV	Neg	I
20	No	No	No	Neg	Neg	Neg	HR+	III	HR+	I
21	No	No	Yes	ASCUS	Neg	Neg	LR+	II	Neg	I
22	No	No	No	ASCUS	Neg	Neg	HR+	III	HR+	I
23	Yes	Yes	No	L-SIL	Pos	Neg	HR+	IV	HR+	I
24	No	No	Yes	L-SIL	Neg	Neg	HR+	III	HR+	IV
25	No	No	Yes	ASCUS	Neg	Neg	LR+	IV	Neg	I
26	Yes	Yes	No	L-SIL	Neg	Neg	HR+	IV	HR+	I
27	Yes	Yes	No	ASCUS	Pos	Neg	HR+	IV	HR+	I
28	Yes	No	No	L-SIL	Neg	Pos	HR+	IV	Neg	I
29	Yes	Yes	No	L-SIL	Neg	Neg	HR+	IV	Neg	I
30	Yes	Yes	Yes	L-SIL	Neg	Neg	HR+	IV	HR+	I
31	Yes	Yes	No	ASCUS	Neg	Neg	HR+	IV	Neg	I
32	No	Yes	No	L-SIL	Neg	Neg	LR+	IV	Neg	I
33	No	No	No	Neg	Neg	Neg	LR+	III	Neg	I
34	No	Yes	Yes	L-SIL	Neg	Neg	HR+	IV	Neg	I
35	No	No	No	L-SIL	Neg	Neg	LR+	III	Neg	I

HPV: Human Papilloma Virus; ASCUS: atypical squamous cells of undetermined significance; L-SIL: low grade squamous intraepithelial lesion;
HR: high rate; LR: low rate; CST: community state type; Pap Test: Papanicolaou test; Neg: negative; Pos: positive.

coxon signed-rank test and sign test (Table I). In order to quantify the strength of the association between the CST and HPV status (Tables I) we used the Kendall rank correlation coefficient. For all the statistical analysis within our study, the α level was set at 0.05.

Results

Features of the enrolled patients

Out of the 40 HPV-positive enrolled patients, 35 (age: 40.0±8.4 years; minimum: 20; maximum: 54) were considered eligible according to the inclusion/exclusion criteria. As seen in Table I, out of 35 women, 27 (77%) tested positive for HR strains (9 women with HPV16; 3 with HPV18; 3 with HPV66; 3 with HPV68; 2 with HPV58; 2 with HPV45; 2 with HPV53; 1 with HPV51; 1 with HPV52; 1 with HPV35) and 8 (23%) tested positive for LR strains. With respect to HPV RNA, 6% of enrolled patients (2/35) tested positive (both women infected with HPV16) and 94% (33/35) tested negative. Regarding colposcopy assessment, 86% (30/35) of the eligible

women showed negative results and 14% (5/35) yielded positive findings, and by biopsy, were shown to be affected by chronic cervicitis. Out of 35 eligible patients, 24 (69%) were characterized by a clear paucity of *Lactobacillus* spp. (CST IV) and of the 11 (31%) with a *Lactobacillus* profile, 10 showed a dominance of *L. iners* (CST III) and 1 a dominance of *L. gasseri* (CST II). Among the women testing positive for HR-HPV, 21 were smokers (Fisher: P=0.002) and 6 were not; 22 used contraceptives (Fisher: P=0.006) and 5 did not. Among women testing positive for LR-HPV, 1 was a smoker and 7 were not; 2 used contraceptives and 6 did not. Out of 24 women characterized as having CST IV status, 21 (87.5%) were smokers (Fisher: P=0.0001) and 3 (12.5%) were not. Out of 11 women with a *Lactobacillus*-dominated microbiota, 1 (9%) was a smoker and 10 (91%) were not (Table I). Among women with CST IV status, contraceptives (Fisher: P=0.0001) were used by 22 (92%) and not used by 2 (8%). Among non-CST IV-classified women, contraceptives were used by 2 (18%) and were not used by 9 (82%). With respect to CST status and Pap test outcome, out of 24 women with CST IV status, 7 (29%) had negative findings for the Pap test and showed only signs of inflammation and hyperkeratosis, while 17 (71%) had positive, but not statistically significant (Fisher: P=0.48), findings (with an ASCUS or L-SIL diagnosis); out of 11 non-CST IV-classified women, 4 (36%) had negative findings for the Pap test (inflammation and hyperkeratosis), while 7 (64%) had positive findings (ASCUS or L-SIL). Out of 24 women with CST IV status, 46% demonstrated signs and/or symptoms of bacterial vaginosis; 54% did not. Out of 27 women testing positive for HR-HPV, 22 (81%) had CST IV status (Kendall rank correlation coefficient, KRCC: 0.51 P=0.003) and 5 (19%) did not. Out of 8 women testing positive for LR-HPV, 2 (25%) had CST IV status and 6 (75%) did not.

Vaginal microbiota and HPV DNA status after 90 days of oral treatment *with L. crispatus* M247

As shown in Table I, after 90 days of oral treatment with *L. crispatus* M247 only 1 woman still demonstrated a vaginal microbiota with very poor *Lactobacillus* spp. content (CST IV) and 34 women were shown to have a vaginal microbiota that was *Lactobacillus*-dominated. Of the non-CST IV-classified women, 33 had a vaginal microbiota that was dominated by *L. crispatus* (CST I) and 1 by *L. iners* (CST III). With respect to richness, the OTU value for the enrolled women was 35±9. After 90 days of treatment, this decreased to 18±5, a statistically significant difference (P<0.01) (data not shown). Regarding HPV DNA status, after 90 days of oral treatment with the probiotic strain, 25 women (71%) tested negative for HR- and LR-HPV, and 10 women (29%) tested positive for HR-DNA (4 women with HPV16; 2 with HPV53; 2 with HPV66; 1 with HPV68; 1 with HPV51). The strength of the association between the CST and HPV status shows the significant effect of having a CST I vaginal microbiota, where only approximately 20% of women were HPV-positive, *versus* having a CST III or CST IV vaginal microbiota, where all women tested positive for HPV (KRCC: 0.389; P=0.02).

Adherence to therapy, tolerability, and unwanted effects due to treatment

According to the patient reports (data not shown), adherence to therapy was greater than 95% and the product was well tolerated with no significant side effects occurring during the 90 days of administration. Some disturbances, such as meteorism and flatulence, were reported by 7 patients. These side effects were considered mild and transient, and after the first two weeks of treatment, were not reported by any patient.

Discussion

Although the number of women assessed was low (N.=35), the characteristics of the enrolled women who were HPV-positive (smoking habits, contraceptive use, and CST status) were significant and in agreement with published data.[41-43] Similarly, those features correlating with CST status (smoking habits and contraceptive use) were significant and in agreement with published data.[44, 45] Finally, the distribution of the HR and LR strains of HPV were in agreement with published data (Table I).[46] After 90 days of treatment with *L. crispatus* M247, 34 out of 35 women

(97%) had a vaginal microbiota that was *Lactobacillus*-dominated and just one woman had a CST IV microbiota (3%). At enrollment this woman had a CST III microbiota. Since sampling for this individual at *t*=90 was carried out on the last day of menses, a time when a certain instability of the vaginal microbiota can be assumed, the sampling time could have negatively affected the result. Moreover, out of 34 women with a *Lactobacillus*-dominated vaginal microbiota at *t*=90, 33 had a microbiota that was *L. crispatus*-dominated (CST I) and one had a microbiota dominated by *L. iners* (CST III).

Regarding HPV status, after 90 days of treatment with strain M247, 71% of women were HPV-negative, with respect to both HR and LR strains, and 29% were still infected by HR-HPV strains (Table I). The two women positive for HPV16 and for mRNA (Table I) showed persistence of infection despite the use of the probiotic strain. Out of nine women testing positive for HPV16, five become HPV-free after treatment. All three women testing positive for HPV18 were negative after treatment. Analyzing the ages of women who were still HR-HPV positive after the treatment, an insignificant distribution can be observed since two women were aged between 20 and 30, four were aged between 31 and 40, and four were aged between 41 and 50. Therefore, even if only for a sample of 35 women, both the vaginal microbiota and HPV status were significantly different from enrollment after the oral use of strain M247. It is possible that these two phenomena could also occur in the absence of therapies and spontaneously, but since epidemiology and meta-analysis studies demonstrate that cervicovaginal *Lactobacillus* spp. are associated with decreased detection of HPV infection and that *L. crispatus* "may be the critical protective factor,"[47] we can consider the possibility that our results may be due to treatment with the probiotic containing *L. crispatus* M247.

Many features of this *Lactobacillus* strain could indeed explain our results. *L. crispatus* M247 was isolated in 1989 from the feces of a healthy baby. Its genome is 2,112,063 bp long and it has 2187 coding and 55 ribosomal genes.[28] The most abundant genes are those involved in carbohydrate metabolism (236 genes), followed by protein metabolism (203), DNA (107) and RNA (84) genes, and genes involved in cell wall and capsule biosynthesis (83). The strain appears to be safe as it does not demonstrate virulence factors and is unlikely to contain plasmids.[28] From a phenotypic perspective, data confirm that the strain is responsive to all tested antibiotics, according to EFSA guidance, and shows a non-transferable resistance to metronidazole and sulfamethoxazole and insensitivity to boric acid.[29] Analysis performed using CRISPRFinder identified four possible CRISPR (clustered regularly interspaced short palindromic repeats) in its genome; two of these CRISPR have been confirmed, while the other two are still under investigation. Genetic analysis also identified a gene encoding for *Lactobacillus* epithelium adhesin (LEA).[28] This gene, previously characterized in *L. crispatus* ST1, was shown to play a major role in vaginal epithelium colonization and in determining competition with *Gardnerella vaginalis*.[48, 49] Moreover, two fibronectin-associated genes (encoding fibronectin type III domain and fibronectin-binding protein A N-terminus; FbpA) were identified in strain M247. These genes have been associated with the ability to colonize the vaginal mucosa and contribute to controlling *G. vaginalis* through a competitive colonization mechanism, thereby reducing the occurrence of bacterial vaginosis.[49] The production of bacteriocins is an important feature of beneficial bacteria in terms of helping to combat pathogenic bacteria. At the gene level, several bacteriocins have been identified in strain M247: two helveticins, one penocin, two enterolysins, and one bacteriocin of the LS2 group. These results are in agreement with previous analyses of other *L. crispatus* genomes and support the possible exploitation of the strain for the control of microbial pathogens.[49] Although the phenotypic presence of bacteriocins released by M247 has not yet been demonstrated, antagonism against some uropathogenic strains of *Escherichia coli* has been observed as well as against *Staphylococcus epidermidis*.[28] As is well known, lactobacilli strains can produce different types of exopolysaccharides (EPS), facilitating bacterial adhesion and protecting against antibiotics and drying.[50] Regarding strain M247, 18 genes encoding enzymes of the glycosyltransfer-

ase group, which are involved in the biosynthesis of EPS, were identified. This suggests that, in suitable environmental conditions, strain M247 can produce EPS.[28] With respect to its probiotic features, when strain M247 is stressed in gastric juice (pH 3; 90 minutes), bile salts (0.5%; 48 hours), and in the pancreatin tolerance test (approximately 0.2%; 3 hours), there is a 2-log reduction in acid conditions, a 1- or 2-log reduction in bile medium (depending on the bile salts used), and there is a 1-log increase in experimental pancreatic juice. Regarding its adhesion properties, strain M247 adhered far better, eight times more, to human ileostomy glycoproteins and to Caco2 cells (a model of colonic epithelium) than its spontaneous isogenic non-aggregating mutant (MU5). Administered in double-blind conditions to healthy volunteers at a dose of at least 10 billion live cells for eight consecutive days, the strain was shown to be a strong colonizer as it was found in all fecal samples and in most biopsy samples.[27] Hydrogen peroxide production by vaginal lactobacilli was long thought to be an important defense mechanism against vaginal colonization by undesirable microorganisms. The production and release by *L. jensenii* strains is especially robust, while this varies among *L. crispatus* and *L. gasseri* strains.[51] M247 has been shown experimentally to be a very good producer of hydrogen peroxide.[28] It has been recently observed that *L. crispatus* M247 also uses hydrogen peroxide as a signal transducing molecule to induce PPAR-γ activation in intestinal epithelial cells, directly modulating epithelial cell responsiveness to inflammatory stimuli.[22] The same mechanism increases the amount of TLR-2 while reducing the amount of TLR-4,[24] thereby improving the strength of tight junctions and reducing the ability of Gram-negative LPS-endowed bacteria to determine TNF-α-mediated inflammatory processes. This anti-inflammatory action exerted by M247 after colonization is evident when tested in a murine model of experimental colitis where the strain can reduce most signs and symptoms of the condition.[25]

As lactobacilli can be transferred from the gut to the vagina, it is thought this is also possible following probiotic administration of a mixture of lactobacilli. The literature indicates that oral administration of lactobacilli is required for 4-5 weeks before any effects on the vagina are seen.[52] Our group recently evaluated the ability of strain M247 to colonize the vagina after oral administration of 20 billion CFU to healthy women. The results obtained following PCR analysis showed that strain M247 was present in the colon of 70% of treated subjects and in the vagina of 40% after only two weeks of oral administration.[28] The probiotic features of *L. crispatus* M247 described above, along with its ability to colonize the colon and vagina after oral administration and its demonstrated antagonism towards some putative vaginal pathogens, suggest that the results observed in our study, although not controlled, could be due to a role played by the bacterial strain administered orally in structuring a *Lactobacillus*-dominant vaginal microbiota capable of promoting an increase in viral clearance.

The CST shifts observed between enrollment and t=90 were significant. It is difficult to evaluate whether the extent of HPV negativization was significant compared to the reported values for spontaneous occurrence. Rodríguez *et al*.[53] reported in 2008 that spontaneous HPV clearance could occur in approximately 20-30% of cases in three months, in approximately 50% in six months, and in approximately 60-70% in one year. More recently, Petry *et al*.[54] reported a spontaneous clearance of virus in approximately 50% of cases in 12 months. We observed a clearance of more than 70% in a period far shorter than one year. Although we are unable to trace the real time of the diagnosis of HPV infection for all 35 women enrolled in our study, these women were either newly diagnosed or with a diagnosis made a few weeks earlier and certainly less than 12 months. This would seem to suggest a treatment-induced effect on the increased viral clearance.

Our study was not organized to interpret the possible results in terms of a mechanism of action; in this regard we can only take a speculative approach, assuming (for instance) a correlation between the *L. crispatus* colonization event and a possible improvement of local immunity against the virus. Similarly, we can assume that some factors produced by the strain could limit the replicative cycle of the virus. Both mechanisms are

plausible, and some authors have clearly reported both a possible effect on immunity due to the presence of *L. crispatus* and a distinct antiviral role played especially by the culture supernatants of *L. crispatus*.[55, 56]

Limitations of the study

Our study has some limitations; these include not being randomized or controlled, not having a placebo group, the sample size, and not having a follow-up to measure the stability of the obtained results. However, our results are, to our knowledge, the first that appear to demonstrate the possibility of intervening with an *L. crispatus* probiotic strain to modify non-*Lactobacillus* (and non-*L. crispatus*) vaginal CSTs to *L. crispatus*-domination, which apparently shows a link to increased HPV clearance.

Conclusions

In conclusion, these results are preliminary and uncontrolled, but they have prompted us to start enrolling a higher number of HPV-positive women to verify (after randomization and in a double-blind and placebo-controlled modality) whether the administration of *L. crispatus* M247 could produce in a long-term manner the biological effects that we have apparently observed here.

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