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Development of a Microarray-Based Tool To Characterize Vaginal Bacterial Fluctuations and Application to a Novel Antibiotic Treatment for Bacterial Vaginosis

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The healthy vaginal microbiota is generally dominated by lactobacilli that confer antimicrobial protection and play a crucial role in health. Bacterial vaginosis (BV) is the most prevalent lower genital tract infection in women in reproductive age and is characterized by a shift in the relative abundances of *Lactobacillus* spp. to a greater abundance of strictly anaerobic bacteria. In this study, we designed a new phylogenetic microarray-based tool (VaginArray) that includes 17 probe sets specific for the most representative bacterial groups of the human vaginal ecosystem. This tool was implemented using the ligase detection reaction-universal array (LDR-UA) approach. The entire probe set properly recognized the specific targets and showed an overall sensitivity of 6 to 12 ng per probe. The VaginArray was applied to assess the efficacy of rifaximin vaginal tablets for the treatment of BV, analyzing the vaginal bacterial communities of 22 BV-affected women treated with rifaximin vaginal tablets at a dosage of 25 mg/day for 5 days. Our results showed the ability of rifaximin to reduce the growth of various BV-related bacteria (*Atopobium vaginae*, *Prevotella*, *Megasphaera*, *Mobiluncus*, and *Sneathia* spp.), with the highest antibiotic susceptibility for *A. vaginae* and *Sneathia* spp. Moreover, we observed an increase of *Lactobacillus crispatus* levels in the subset of women who maintained remission after 1 month of therapy, opening new perspectives for the treatment of BV.

The human body harbors an enormous number of microorganisms that inhabit surfaces and cavities exposed or connected to the external environment (1). As one of these human-microbe habitats, the female genital tract is inhabited by bacterial communities that are known to confer antimicrobial protection to the vagina and play a crucial role in health (2, 3). The healthy vaginal microbiota is generally dominated by at least one *Lactobacillus* sp. among *L. crispatus*, *L. iners*, *L. jensenii*, and *L. gasseri* (4). Alterations in the types and relative proportions of the microbial species in the vagina can be associated with the development of infectious conditions, such as bacterial vaginosis (BV), aerobic vaginitis (AV), candidiasis (CA), and sexually transmitted infections (STI) (5, 6).

BV is the most prevalent lower genital tract infection in women in reproductive age (7) and is associated with several adverse obstetrical and gynecological outcomes and increased risk for acquisition of HIV (8–10). BV is characterized by a shift in the relative abundances of *Lactobacillus* spp. to a greater abundance of strictly anaerobic bacteria, including *Gardnerella vaginalis*, *Atopobium vaginae*, *Mycoplasma hominis*, and species belonging to *Prevotella*, *Mobiluncus*, *Megasphaera*, *Sneathia*, and *Eggerthella* genera (6, 11–13). BV is usually treated with antibiotics, including metronidazole and clindamycin (14); however, relapse rates are high and factors leading to relapse are poorly understood (15).

Rifaximin, a broad-spectrum antibiotic with low systemic absorption, traditionally used for the treatment of numerous gastrointestinal diseases (16), has been recently proposed as a new therapeutic agent for the cure of BV (17). The analysis of the clinical parameters (17), the molecular composition of vaginal communities (18), and the proteomic and metabolic profiles of vaginal fluids (19, 20) revealed that treatment with 25 mg of rifaximin for 5 days can effectively counteract the alterations associated with the BV condition.

Here we developed a new DNA-microarray platform, named VaginArray, for fast, reliable, and low-cost analysis of the variations of the most representative bacterial groups that compose the vaginal microbiota. The VaginArray was implemented using the ligase detection reaction-universal array (LDR-UA) approach (21–23). LDR is based on the discriminative properties of the DNA ligase and requires the design of a pair of adjacent probes specific for each target: a 5′-fluorophore modified oligonucleotide (discriminating probe [DS]) and a second probe (common probe [CP]), starting one base 3′ downstream of the DS and carrying a 5′ phosphate group and a unique sequence named cZipCode at its 3′ end. The probe pair mix and a thermostable DNA ligase are used

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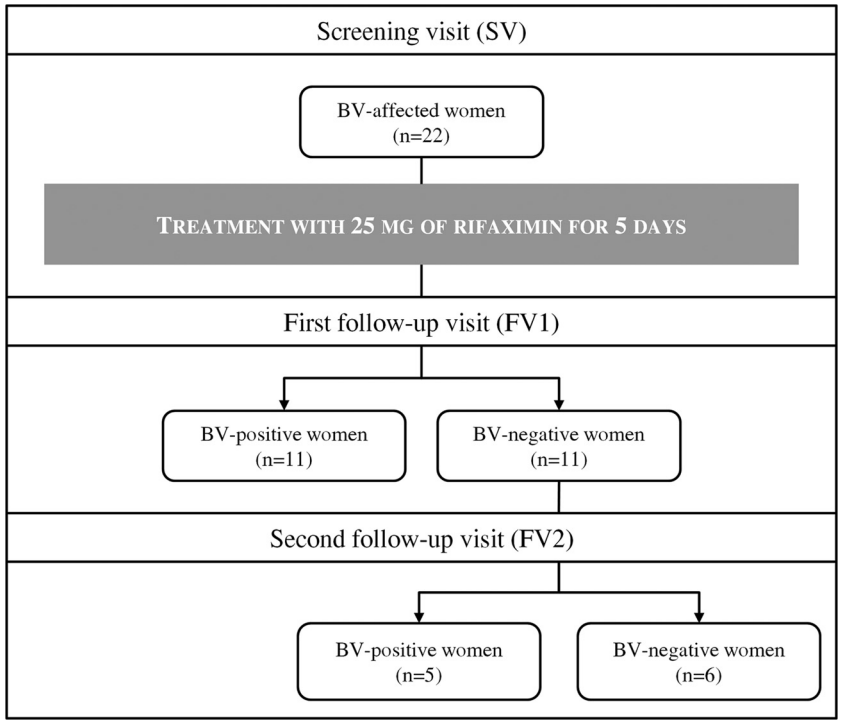

FIG 1 Design of the clinical study used as a validation model of the VaginArray.

in a thermal cyclic reaction with PCR amplicons as the templates. LDR products form only in the presence of a perfectly matching template-DS-CP complex and are addressed to a precise location on a universal array (UA) where a set of artificial sequences (i.e., sequences designed in order to have no interaction to any known sequence) called ZipCodes, complementary to the cZipCodes, are arranged. LDR products, carrying both the fluorescent label and the unique cZipCode, are detected by laser scanning and identified according to their location on the array. The VaginArray was successfully tested and validated in a clinical study aimed at assessing the efficacy of vaginal rifaximin tablets for the cure of BV.

MATERIALS AND METHODS

Target selection and consensus extraction. A database of 16S rRNA gene sequences of selected bacterial groups of particular interest for the vaginal microbial ecosystem was created by using sequences available in the Ribosomal Database Project (RDP; release 10, 7 December 2012) (http://rdp.cme.msu.edu/) (24). Single species, groups of phylogenetically related species, or genera belonging to the human vaginal microbiota were rationally selected based on the available literature (4, 6, 12, 13). A phylogenetic tree based on the 16S rRNA sequences used for probe design was created by using MEGA 6 software (25). Group-specific consensus sequences were extracted, with a cutoff value of 75% for base calling. Nucleotides which occurred at lower frequencies were replaced by the appropriate International Union of Pure and Applied Chemistry (IUPAC) ambiguity code. At the same time, a "negative" set was built, including the sequences of species the specific targets had to differ from.

Probe design. Multiple alignment of the selected sequences was performed in ClustalW (26). All the LDR probe pairs were designed using ORMA software (27), following the procedure used for the HTF-MicroBi.Array (21). Briefly, for each target, the appropriate consensus sequence was used to identify positions capable of discriminating it from other target and nontarget sequences (i.e., sequences from the positive set other than the tested one and those belonging to the negative set, respectively). A discriminating base is defined as a single nucleotide peculiar to a single consensus sequence in the whole data set (i.e., both the positive and negative sets) and, thus, capable of specifically distinguishing that sequence from all the others in an enzyme-mediated ligation reaction. Both the DS and CP were required to be between 25 and 60 base pairs in length, with a melting temperature (T_m) of 68 ± 1°C and with maximum of 4 degenerated bases; moreover, we required degenerated bases to be at least 5 bases from the edge of each probe (i.e., from the 3′ of the DS or from the 5′ of CP) and at least 4 bases apart from each other. *In silico* checks against the publicly available RDP database were performed for assessing probe pair specificity.

Subject recruitment and samples. A total of 22 European premenopausal, nonpregnant women were selected to test the VaginArray. These women belonged to a specific treatment arm in a clinical study designed to assess the efficacy of rifaximin vaginal tablets for the treatment of BV (EudraCT number 2009-011826-32) (17). At the screening visit (SV), the women were diagnosed with BV as they presented a Nugent score of >3 and were positive for at least three of Amsel's criteria. The patients received a daily rifaximin vaginal tablet of 25 mg for 5 days, which was administered intravaginally at bedtime. The patients attended a first follow-up visit (FV1) 7 days after the end of the therapy. Only the patients showing remission at FV1 attended a second follow-up visit (FV2) 28 days after the end of the treatment (Fig. 1). Diagnosis of remission at both follow-up visits was made for women presenting with a Nugent score of ≤3 and positive for 2 or fewer of Amsel's criteria. Informed consent was obtained from all subjects in accordance with the local Ethics Committees. Standardized vaginal rinsings with 2 ml of saline solution were collected for molecular studies by flushing and reaspirating the fluid through a 22-gauge needle in the left, central, and right upper vaginal vaults as described elsewhere (28, 29). The vaginal samples were subsequently stored at −80°C and used for DNA extraction within 2 months. This study was approved by the Institutional Review Board.

DNA preparation. Bacterial DNA from *Atopobium vaginae* DSM15829, *Eggerthella lenta* DSM2243, *Gardnerella vaginalis* DSM4944, *Lactobacillus crispatus* DSM20584, *L. iners* DSM13335, *L. jensenii* DSM20557, *L. vagi-*

nalis DSM5837, *Leptotrichia buccalis* DSM1135, *Megasphaera elsdenii* DSM20460, *Mobiluncus curtisii* DSM2711, *Mycoplasma hominis* DSM19104, *Prevotella bivia* DSM20514, *Sneathia amnii* DSM16630, *S. sanguinegens* DSM22970, *Streptococcus agalactiae* DSM2134, and *Veillonella parvula* DSM2008 was directly obtained from the DSMZ (Braunschweig, Germany).

Genomic DNA from *Lactobacillus acidophilus* DSM20079, *L. gasseri* DSM20243, and *Staphylococcus aureus* ATCC 12600 was extracted from 10^9 bacterial cells by using a DNeasy blood and tissue kit (Qiagen, Düsseldorf, Germany) following the manufacturer instructions. *Lactobacillus* strains were grown on De Man-Rogosa-Sharpe (MRS) broth with cysteine (0.5 g/liter) at 37°C, under an anaerobic atmosphere (Anaerocult; Merck, Darmstadt, Germany). *S. aureus* ATCC 12600 was grown at 37°C aerobically on Luria-Bertani (LB) broth.

Total bacterial DNA was extracted from vaginal rinsings by using a DNeasy blood and tissue kit (Qiagen) as previously described (18).

Genomic DNAs extracted from bacterial cultures and vaginal fluids were quantified using a NanoDrop ND-1000 instrument (NanoDrop Technologies, Wilmington, DE).

PCR. All the oligonucleotide primers and probe pairs were synthesized by Thermo Fisher Scientific (Ulm, Germany). PCR amplifications were performed with a Biometra Thermal Cycler II instrument (Biometra, Germany). 16S rRNA was amplified using universal forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), following the protocol described by Candela et al. (21), with slight modifications. Briefly, the reaction mixture included a 0.5 μM concentration of each primer, a 200 μM concentration of each deoxynucleoside triphosphate (dNTP), 2 mM $MgCl_2$, 2.5 U of Gotaq Flexy polymerase (Promega, Madison, WI), and 50 ng of genomic DNA in a final volume of 50 μl. Prior to amplification, the DNA was denatured for 5 min at 95°C. Amplification consisted of 40 cycles at 95°C for 60 s, 60°C for 30 s, and 72°C for 90 s. After the cycles, an extension step (5 min at 72°C) was performed. PCR products were purified by using a High Pure PCR Clean up Micro kit (Roche, Mannheim, Germany), following the manufacturer instructions, eluted in 20 μl of sterile water, and quantified with the NanoDrop ND-1000 instrument.

LDR-UA approach. Phenylen-diisothiocyanate (PDITC) activated chitosan glass slides were used as surfaces for the preparation of universal arrays (30), comprising a total of 49 ZipCodes. Hybridization controls (cZip 66 oligonucleotide, complementary to zip 66 [5′-Cy3-GTTACCGCTGGTGCTGCCGCCGGTA-3′]) were used to locate the submatrices during the scanning. The entire experimental procedure for both the chemical treatment and the spotting is described in detail by Consolandi et al. (31). An overview of the UA layout and ZipCodes is provided as Fig. S1 in the supplemental material. Ligase detection reactions and hybridization of the products on the universal arrays were performed according to the protocol of Castiglioni et al. (22), except for the probe annealing temperature, which was set at 60°C. The LDRs were carried out in a final volume of 20 μl with different quantities of purified PCR products: (i) all LDRs for specificity tests were performed on 10 ng of initial PCR product; (ii) sensitivity tests were performed with amounts of PCR product decreasing from 96 ng to 6 ng; and (iii) LDR experiments on human vaginal samples were performed on 48 ng of PCR product. In all experiments, 250 fmol of synthetic template (5′-AGCCGCGAACACCACGATCGACCGGCGCGCGCAGCTGCAGCTTGCTCATG-3′) was used for normalization purposes.

qPCR analysis. Quantitative PCR (qPCR) was performed on DNA samples extracted from the vaginal fluids using a LightCycler instrument (Roche, Mannheim, Germany) and SYBR green I as the reporter fluorophore. *G. vaginalis* was amplified by using the species-specific primer set F-GV1/R-GV3 (32). Amplifications were carried out in a final volume of 20 μl containing each primer at 0.5 μM, 4 μl of LightCycler-FastStart DNA Master SYBR green I (Roche), and 2 μl of the template. The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 15 s; primer annealing at 60°C for 20 s; extension at 72°C for 45 s; and a fluorescence acquisition step at 85°C for 5 s. DNA extracted from *G. vaginalis* DSM 4944 was used as the standard for PCR quantification. DNAs extracted from vaginal samples were amplified in triplicate. Data were expressed as ng of DNA of *G. vaginalis* per μg of total DNA extracted from the vaginal sample.

Data analysis. Arrays were scanned using a ScanArray 5000 scanner (PerkinElmer Life Sciences, Boston, MA, USA) at 10-μm resolution, and the fluorescence intensity (IF) was quantitated by ScanArray Express 3.0 software, as described by Candela et al. (21). In order to be able to compare data from different samples, a normalization procedure based on the IFs of the synthetic ligation control signal was applied as follows: (i) outlier values (2.5-fold above or below the average) were discarded; (ii) a correction factor was calculated in order to set the average IF of the ligation control to 50,000 ($n = 6$); and (iii) the correction factor was applied to both the probes and the background IF values. Statistically significant probe pair results were determined using a one-sided *t* test comparing, for each ZipCode, the distribution of IFs along all replicates with the distribution of IFs of negative controls (i.e., "blanks," where only printing buffer has been spotted).

Hierarchical clustering of the VaginArray profiles was carried out using R statistical software (http://www.r-project.org). Ward's method was used for agglomeration. The prevalence for each bacterial group was calculated as the percentage of the patients showing a significant mean IF value, determined as described above, for the considered probe.

Statistical analysis was performed using SigmaStat (Systat Software, Point Richmond, CA). Chi-square analysis of contingency was used to test the significance of the differences found in the prevalence rates in the cluster analysis, in the fluorescence signals, and in the qPCR data. Differences in the amounts of target bacteria belonging to the clusters identified by hierarchical clustering were analyzed by the Mann-Whitney U test, while differences determined by fluorescence signals and qPCR data were analyzed using Wilcoxon's signed-rank test. A P value of <0.05 was considered the threshold for significance in all the tests.

RESULTS

Target selection and probe design. Seventeen bacterial targets were rationally selected (Table 1) based on the available literature (4, 6, 12, 13), with the aim to develop a tool for the detection of the most representative species of the human vaginal ecosystem, under both healthy and infection conditions, especially in cases of BV. A primary objective of the VaginArray was to characterize the *Lactobacillus* population at the level of the most representative single species or small groups of close phylogenetic relatives. To this aim, first, 6 probes targeting *L. rispatus*, *L. iners*, *L. vaginalis*, *L. acidophilus*, *L. gasseri* and related species (et rel.) (*L. gasseri* and *L. johnsonii*), and *L. jensenii* et rel. (*L. jensenii* and *Lactobacillus fornicalis*) were designed. Second, in order to obtain a more complete view of the vaginal ecosystem under BV conditions, the most reported species or genera associated with the altered ecosystem typical of BV were also targeted. Ten probes were designed for the species *Atopobium vaginae* and *Mycoplasma hominis*, the *Sneathia* group (*S. sanguinegens* and *S. amnii*, formerly *Leptotrichia amnionii*) (33), and species of the genera *Streptococcus*, *Staphylococcus*, *Veillonella*, *Megasphaera*, *Mobiluncus*, *Leptotrichia* and *Eggerthella*. In order to detect the presence of bacteria belonging to the genus *Prevotella*, which is also often associated with BV status, the *Bacteroides*/*Prevotella* probe from the HTF-MicroBi.Array (21) was added to the VaginArray. The plethora of vaginal microorganisms targeted by the VaginArray is showed by the phylogenetic tree obtained from the entire positive sequence sets used for the probe design (Fig. 2). Specificity and coverage of each candidate probe were assessed by using the tool Probe Match of the RDP

TABLE 1 Probe sets of the VaginArray

Probe	Taxonomic level	Phylum	Specificity	Sensitivity (ng)
Lactobacillus vaginalis	Species	*Firmicutes*	*L. vaginalis*	6
Lactobacillus iners	Species	*Firmicutes*	*L. iners*	6
Lactobacillus crispatus	Species	*Firmicutes*	*L. crispatus*	12
Lactobacillus acidophilus	Species	*Firmicutes*	*L. acidophilus*	12
Lactobacillus jensenii et rel.	Closely related species	*Firmicutes*	*L. jensenii*, *L. fornicalis*	12
Lactobacillus gasseri et rel.	Closely related species	*Firmicutes*	*L. gasseri*, *L. johnsonii*	6
Streptococcus	Genus	*Firmicutes*	*Streptococcus* sp.	6
Staphylococcus	Genus	*Firmicutes*	*Staphylococcus* sp.	6
Veillonella	Genus	*Firmicutes*	*Veillonella* sp.	6
Megasphaera	Genus	*Firmicutes*	*Megasphaera* sp.	6
Bacteroides/Prevotella	Cluster	*Bacteroidetes*	*Prevotella* sp.	6
Mobiluncus	Genus	*Actinobacteria*	*Mobiluncus* sp.	6
Atopobium vaginae	Species	*Actinobacteria*	*A. vaginae*	6
Eggerthella	Genus	*Actinobacteria*	*Eggerthella* sp.	12
Sneathia	Genus	*Fusobacteria*	*S. sanguinegens*, *S. amnii* (formerly *Leptotrichia amnionii*)	6
Leptotrichia	Genus	*Fusobacteria*	*Leptotrichia* sp.	6
Mycoplasma hominis	Species	*Tenericutes*	*M. hominis*	12

database. The designed probe pairs had an average T_m of 67.6 ± 1.5°C (n = 34) and a length of between 25 and 48 nucleotides (Table S1 in the supplemental material).

Validation of the VaginArray: specificity and sensitivity. The specificity of the designed LDR probe pairs was tested by using 16S rRNA PCR amplicons from 18 microorganism members of the human vaginal microbiota. Amplicons were prepared by amplification of genomic DNA provided by DSMZ or extracted from pure cultures. All the 16S rRNA amplicons were properly recognized in separate LDR hybridization reactions with the entire probe set of the array (see Fig. S2 in the supplemental material). For each of the 16S rRNA templates, only group-specific spots and spots corresponding to the hybridization controls showed positive signals ($P < 0.0005$). The ratio between specific and nonspecific probes was more than 100-fold on average.

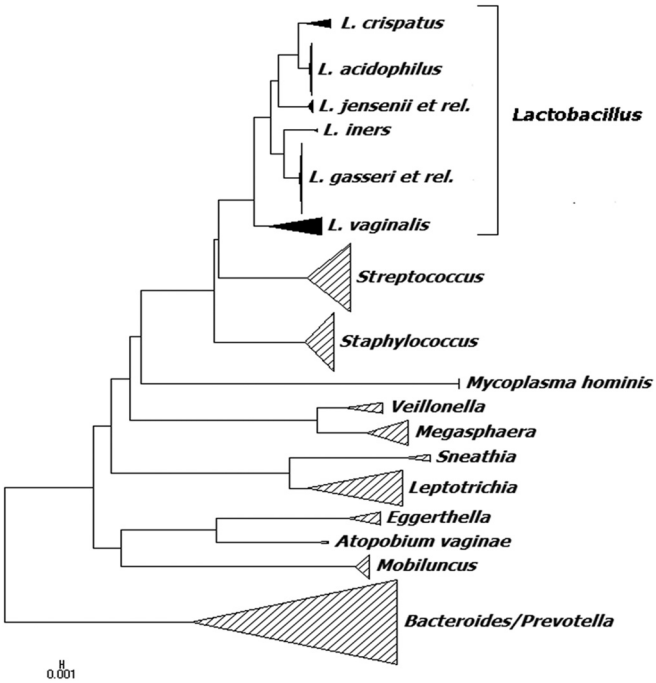

FIG 2 Phylogenetic tree showing the plethora of vaginal microorganisms targeted by the VaginArray. The tree was obtained from the entire positive-testing sequence sets used for the probe design. The neighbor-joining method was used to infer evolutionary history. The evolutionary distances were computed using the maximum composite likelihood method and are quantified in units of the number of base substitutions per site. The analysis involved 573 nucleotide sequences. All positions containing gaps and missing data were eliminated. The tree was obtained by using MEGA6 software (25).

In order to define the detection limits of the VaginArray, LDR-UA experiments were carried out with different concentrations of artificial mixes containing equal amounts of 16S rRNA amplicons from the target bacteria. The 16S rRNA amplicons were all specifically recognized in a range of total DNA amounts from 96 to 12 ng ($P < 0.0005$), corresponding to approximately 10^6 bacterial cells, considering the molecular weight of the *Escherichia coli* genome as a reference (Table 1). Determination of sensitivity for six representative members of the human vaginal microbiota (*L. acidophilus*, *L. crispatus*, *L. jenseni*, *M. hominis*, *P. bivia*, and *S. agalactiae*) is reported as an example in Fig. S3 in the supplemental material.

Clinical validation of the VaginArray: application to rifaximin treatment of BV. The new tool VaginArray was validated on 55 clinical samples from 22 BV-affected women treated with rifaximin vaginal tablets at the dosage of 25 mg/day for 5 days. Demographic information for the women included in this study are listed in Table 2. Therapeutic remission was observed in 11/22 (50%) patients at the first follow-up visit (FV1) and was maintained in 6/11 (55%) patients at the second follow-up visit (FV2) (Fig. 1).

Validation of the VaginArray was obtained by comparing the microarray-based identification of the target bacteria with the Nugent score and Amsel's criteria, which are reported in Table S2 in the supplemental material. Dols et al. (34) proposed a similar validation of their vaginal microarray, considering only Nugent scoring. Amsel's criteria were also taken into account in the present work in order to have a more consistent correlation with the diagnosis of BV based on both methods. Percentages of vaginal

TABLE 2 Demographic characteristics of the women analyzed in the study

Characteristic[a]	Mean ± SD or percentage
Age (yrs)	36 ± 9
Caucasian	100%
Weight (kg)	65 ± 15
Height (cm)	167 ± 5
BMI (kg/m^2)	23 ± 5
History of STDs	4%
Previous vaginal intercourse	100%

[a] Abbreviations: BMI, body mass index; STDs, sexually transmitted diseases.

TABLE 3 Microarray identification of vaginal communities, Nugent scoring, and Amsel's criteria[a]

Probe	% of vaginal samples positive for the VaginArray probes			
	Nugent ≤ 3 (n = 17)	Nugent > 3 (n = 38)	Amsel < 3 (n = 24)	Amsel ≥ 3 (n = 31)
L. vaginalis	35	3	25	3
L. iners	77	82	75	84
L. crispatus	47	13	42	10
L. acidophilus	0	0	0	0
L. jensenii et rel.	59	34	54	32
L. gasseri et rel.	12	16	13	16
Streptococcus	12	16	13	16
Staphylococcus	23	16	21	16
Veillonella	29	16	21	19
Megasphaera	47	90	54	94
Bacteroides/Prevotella	77	97	79	100
Mobiluncus	47	68	54	68
Atopobium vaginae	12	84	33	87
Eggerthella	0	0	0	0
Sneathia	12	66	21	71
Leptotrichia	6	8	4	10
Mycoplasma hominis	23	13	17	16

[a] Percentages (%) of vaginal samples positive for the VaginArray probes are indicated in relation to Nugent scores and Amsel's criteria, according to BV diagnosis.

samples positive for the VaginArray probes were calculated in relation to Nugent scores (≤3 or >3) and Amsel's criteria (<3 or ≥3) (Table 3). Our data showed that the microarray-based identification of the main *Lactobacillus* species associated with vaginal health, i.e., *L. crispatus*, *L. jensenii*, and *L. vaginalis*, was inversely correlated with both the Nugent score and Amsel's criteria, while the detection of the major BV-related bacteria, i.e., *Megasphaera*, *Bacteroides*/*Prevotella*, *Mobiluncus*, *A. vaginae*, and *Sneathia* spp., was directly correlated with both parameters used for BV diagnosis. For example, *L crispatus* was detected in 47% and 13% of vaginal fluids with Nugent scores of ≤3 and >3, respectively, and in 42% and 10% of samples with Amsel's criteria of <3 and ≥3, respectively; *A. vaginae* was identified in 12% and 84% of vaginal samples which had Nugent scores of ≤3 and >3, respectively, and in 33% and 87% of samples with Amsel's criteria of <3 and ≥3, respectively.

The natural logarithms of IF signals registered for the microarray probes in relation to the clinical diagnosis (BV positive or BV negative) and the time of sample collection (SV, FV1, or FV2) were compared. Hierarchical clustering based on the log IF data of the target bacterial groups in a heat map identified two main groups of samples, cluster A and cluster B (Fig. 3). The proportion of BV-negative samples in cluster A is significantly higher than in cluster B ($P < 0.001$). Significant differences in clustering ($P = 0.001$) were also observed for samples collected at different visits: SV samples mainly grouped in cluster B in accordance with BV diagnosis, while FV1 and FV2 samples grouped in cluster A or B in relation to the clinical diagnosis. A significantly higher number of samples belonging to cluster A showed the presence of *L. crispatus* and *L. vaginalis* ($P < 0.001$) and the absence of *A. vaginae*, *Megasphaera*, and *Sneathia* spp. ($P < 0.005$) than of samples belonging to cluster B. Moreover, samples in cluster A were characterized by higher IF signals for *L. crispatus* and *L. vaginalis* ($P < 0.001$) and lower signals for *A. vaginae*, *Bacteroides*/*Prevotella*, *Megasphaera*, and *Sneathia* spp. ($P < 0.001$) than samples in cluster B.

Table 4 presents the prevalence rates of each analyzed bacterial group in relation to the different time points of the study. The totality of women enrolled in the study showed a significant drop ($P < 0.001$) in the prevalence rate of *A. vaginae* and *Sneathia* spp. after the antibiotic administration. Among the other infection-related bacteria, a slight reduction in the percentage of women harboring *Bacteroides*/*Prevotella* spp., *Megasphaera* spp., and *Mobiluncus* spp. was observed. No change involved *Veillonella* spp., while a trend toward an increase of prevalence was registered for *M. hominis*. However, the *M. hominis* prevalence remained constant in women who maintained remission at FV2 and no significant change was recorded in quantitative terms, suggesting that the low sensitivity of *M. hominis* to rifaximin (35) did not lead to the overgrowth of this organism as a side effect to antibiotic therapy. These data, highlighting a lack of a visible effect exerted by rifaximin on *Veillonella* and *M. hominis*, are in agreement with the percentages of identification of these bacterial groups in relation to the values of Nugent scores and Amsel's criteria, as reported in Table 3. For the population of lactobacilli, the highest prevalence rate at SV was observed for *L. iners* followed by *L. jensenii*; the prevalences of both species slightly decreased at FV1. Within the subset of women who went into remission at FV1, the bacterial groups whose prevalence rates were reduced at FV1 showed in some cases an increased occurrence at FV2, albeit maintaining lower values compared to the baseline. *A. vaginae* ($P < 0.001$) and *Sneathia* spp. ($P < 0.05$) showed the highest susceptibility to the antibiotic activity, as demonstrated by the low prevalence at FV1 and the partial maintenance of the effect at FV2. Moreover, *Megasphaera* and *Mobiluncus* spp. showed a trend toward a reduction in prevalence after rifaximin treatment. In contrast, the percentages of women positive for *M. hominis* and *Veillonella* spp. increased at FV1 and FV2, respectively. *L. iners* and *L. jensenii* showed the highest prevalence rates among lactobacilli at SV, and those rates were maintained throughout the study. It is noteworthy that *L. crispatus* and *L. vaginalis* showed a trend toward increasing their prevalence rates during the study period. Women who maintained in remission at FV2 showed a reduction in the prevalence of *A. vaginae*, *Megasphaera*, *Mobiluncus*, and *Sneathia* spp. at the follow-up visits, with a significant variation for *A. vaginae* ($P < 0.005$). Among lactobacilli, a tendency to increase in prevalence was observed for *L. jensenii*, *L. crispatus*, and *L. vaginalis*, while *L. gasseri* was not detected at FV2.

In order to further assess the impact of rifaximin on the composition of the vaginal microbiota, log IF values were an-

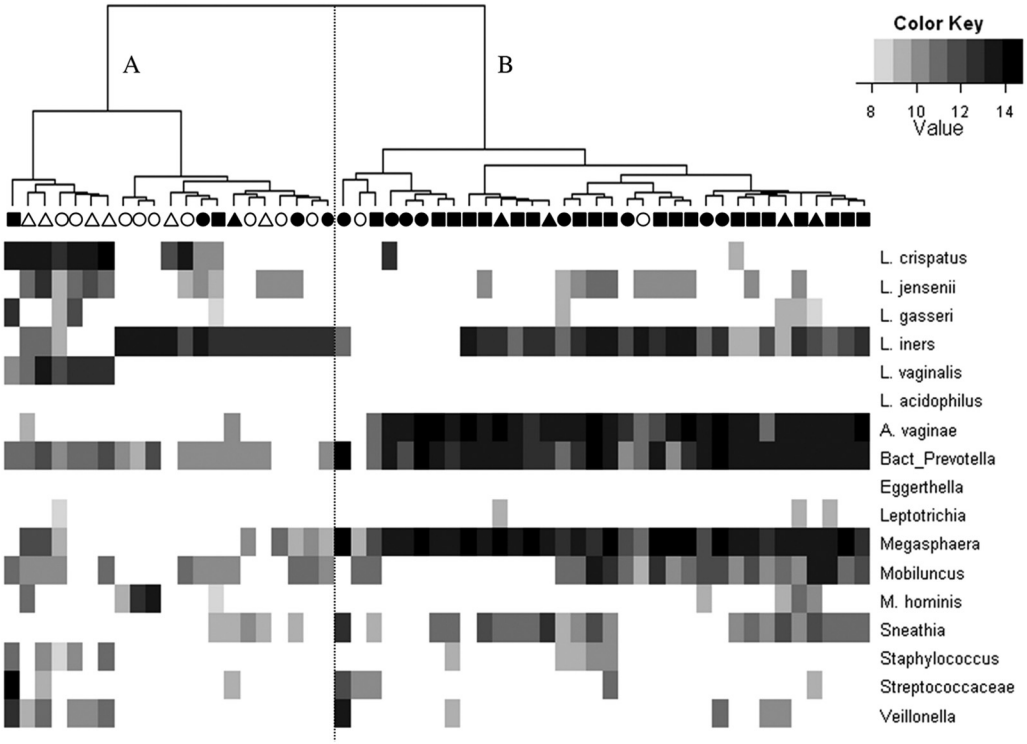

FIG 3 Hierarchical clustering and heat map of the VaginArray data related to the analyzed samples. The dotted vertical line separates the two A and B clusters. The white and black symbols represent BV-negative and BV-positive samples, respectively. The squares, circles, and triangles represent samples collected at the screening visit (SV), the first follow-up visit (FV1) and the second follow-up visit (FV2), respectively. The mean values of the natural logarithm of fluorescence intensity for each bacterial groups are plotted in a scale of gray. Ward's clustering method was used. Bact_Prevotella, bacteria belonging to the genus *Prevotella*.

alyzed to search for quantitative variations of the target bacteria over the course of the study (Table 4). Significant changes were observed for *L. crispatus*, *L. vaginalis*, *A. vaginae*, and *Prevotella*, *Megasphaera*, *Mobiluncus*, and *Sneathia* spp. The analysis of the variations that occurred in all the women enrolled in the study revealed a remarkable drop ($P < 0.001$) in the median log IF values of the considered bacteria after rifaximin treatment, with the exception of *L. crispatus* and *L. vaginalis*, which showed no variation. In considering the subset of women who went into remission at FV1, an increase of the signal was registered for *A. vaginae* and *Prevotella* and *Sneathia* spp. at FV2 ($P < 0.05$), with respect to FV1. However, *A. vaginae* and *Megasphaera* median log IF values at FV2 were still significantly lower than those at SV ($P < 0.02$). The median log IF for the lactobacilli did not change in this group of patients. Interestingly, women who maintained remission at FV2 showed a significant increase of *L. crispatus* and *L. vaginalis* signals at FV2 with respect to SV ($P < 0.05$). *A. vaginae* and *Megasphaera* levels significantly decreased at FV1 and FV2 ($P < 0.01$), while *Prevotella* spp. showed a reduction at FV1 ($P = 0.02$) followed by a slight increase at FV2. *Mobiluncus* and *Sneathia* spp. did not show significant changes, even though the signal tended to decrease at FV1, with maintenance of the effect at FV2.

Variations of *G. vaginalis* associated with rifaximin treatment. Variations of *G. vaginalis* during the study were analyzed by qPCR. Prevalence rates and concentration values of this species are reported in Table 5. For all three sets of women (total women, women in remission at FV1, and women in remission at FV2), we observed a significant reduction in the prevalence rate at FV1 compared to SV ($P < 0.005$). In particular, in women maintaining remission at FV2, *G. vaginalis* prevalence decreased to 0 at both follow-up visits after starting from a value of 83% at the baseline ($P < 0.005$). Regarding the *G. vaginalis* concentration, a significant drop compared to SV was found at both FV1 ($P < 0.005$) and FV2 ($P < 0.05$) in women who were in remission at FV1. Women who maintained remission at FV2 showed no presence of *G. vaginalis* at both FV1 ($P < 0.05$) and FV2 ($P < 0.05$), demonstrating the efficacy of rifaximin treatment in reducing the vaginal colonization of *G. vaginalis*.

DISCUSSION

Comprehensive knowledge of the composition of the vaginal microbiota is essential for understanding the etiology of diverse diseases of the female genital tract and for the development of new diagnostic tools, effective treatments, and prevention protocols (36). In the present work, we have developed and validated the VaginArray, a new phylogenetic DNA microarray designed for monitoring variations in the most relevant bacterial groups of the human vaginal microbiota. Thanks to the implementation with the LDR-UA technology, the VaginArray represents a fast, low-cost, and reliable platform, useful to screen many samples in a short time. The high specificity and sensitivity of this tool allowed us to overcome the major limitations of DNA microarrays whose discriminative power is based on hybridization, as reported in Castiglioni et al. and Hultman et al. (22, 23). Ideal applications of the VaginArray

TABLE 4 Prevalence rates and log IF values of the target bacteria at the different visits of the study (SV, FV1, and FV2)[a]

Subject category	Bacterial group	PR (%)/log IF			Significance	
		SV	FV1	FV2	PR[b]	Log IF[c]
Total women ($n = 22$)	*L. crispatus*	14/4.5	23/5.0	na		
	L. jensenii	46/4.1	36/3.9	na		
	L. gasseri	14/4.3	14/4.0	na		
	L. iners	82/5.5	77/5.4	na		
	L. vaginalis	5/3.2	9/4.3	na		
	L. acidophilus	0/ns	0/ns	na		
	A. vaginae	91/5.9	36/5.5	na	**	**
	Prevotella	100/5.7	82/5.5	na		**
	Leptotrichia	9/3.1	5/2.4	na		
	Megasphaera	91/5.9	73/5.4	na		**
	Mobiluncus	73/5.1	55/4.5	na		**
	M. hominis	9/3.4	18/4.6	na		
	Sneathia	77/4.6	18/4.2	na	**	**
	Staphylococcus	23/3.7	14/3.2	na		
	Streptococcus	14/4.8	9/3.8	na		
	Veillonella	14/4.1	14/4.7	na		
	Eggerthella	0/ns	0/ns	na		
Women in remission at FV1 ($n = 11$)	*L. crispatus*	18/4.8	27/5.3	46/5.5		
	L. jensenii	46/4.1	46/4.1	46/4.7		
	L. gasseri	18/4.6	18/4.3	18/3.2		
	L. iners	82/5.3	82/5.5	82/5.4		
	L. vaginalis	9/3.5	18/4.6	36/5.1		
	L. acidophilus	0/ns	0/ns	0/ns		
	A. vaginae	91/5.7	9/3.9	55/5.5	**	**; #; +
	Prevotella	100/5.6	73/4.6	91/5.4		**; +
	Leptotrichia	9/3.1	9/2.7	9/3.0		
	Megasphaera	91/5.9	55/4.2	55/5.5		**; #
	Mobiluncus	82/4.8	46/4.3	55/4.9		*
	M. hominis	9/3.6	27/4.9	27/4.1		
	Sneathia	73/4.5	9/3.2	55/4.7	*	*; +
	Staphylococcus	18/3.7	18/3.4	18/3.8		
	Streptococcus	18/5.1	9/3.3	27/3.5		
	Veillonella	9/4.3	9/3.5	46/4.3		
	Eggerthella	0/ns	0/ns	0/ns		
Women in remission at FV2 ($n = 6$)	*L. crispatus*	17/5.0	50/5.5	83/5.8		#
	L. jensenii	50/4.1	67/4.3	83/5.0		
	L. gasseri	33/4.9	33/4.5	0/ns		
	L. iners	83/5.4	83/5.4	67/5.4		
	L. vaginalis	17/3.7	33/4.9	67/5.3		#
	L. acidophilus	0/ns	0/ns	0/ns		
	A. vaginae	83/5.7	0/ns	17/3.5	**	*; #
	Prevotella	100/5.6	67/4.5	83/4.9		
	Leptotrichia	17/3.4	17/2.9	0/ns		
	Megasphaera	83/6.0	50/4.1	33/4.7		*; #
	Mobiluncus	83/4.8	50/4.4	50/4.3		
	M. hominis	17/3.9	17/3.4	17/4.2		
	Sneathia	50/4.4	0/ns	17/3.4		
	Staphylococcus	17/3.9	33/3.7	33/4.1		
	Streptococcus	17/5.3	0/ns	17/3.4		
	Veillonella	17/4.6	17/3.7	17/4.5		
	Eggerthella	0/ns	0/ns	0/ns		

[a] PR, prevalence rate; na, not available; ns, no signal registered from VaginArray analysis.
[b] Chi-square analysis of contingency was used to test differences in the prevalence rates of each bacterial group. *, $P < 0.05$; **, $P < 0.005$.
[c] Wilcoxon's signed-rank test was used to test differences in the log IF values of each bacterial group. *, $P < 0.05$ for FV1 versus SV; **, $P < 0.005$ for FV1 versus SV; #, $P < 0.05$ for FV2 versus SV; +, $P < 0.05$ for FV2 versus FV1.

TABLE 5 Prevalence rates and concentration values obtained with qPCR related to *G. vaginalis*[a]

Subject category	PR (%)/log concn (mean ± SD)			Significance	
	SV	FV1	FV2	PR[b]	Log concn[c]
Total women ($n = 22$)	95/2.3 ± 1.5	50/2.4 ± 1.4	na	*	
Women in remission at FV1 ($n = 11$)	10/2.3 ± 1.3	1/−0.02 ± −0.9	4/1.8 ± 0.5	*	**; #
Women in remission at FV2 ($n = 6$)	83/2.4 ± 1.4	0/ns	0/ns	*	*; #

[a] Concentration data are expressed as log ng of *G. vaginalis* DNA per μg of total DNA extracted from the vaginal sample. PR, prevalence rate; na, not available; ns, no signal registered from qPCR analysis.

[b] Chi-square analysis of contingency was used to test differences in the prevalence rates. *, $P < 0.005$.

[c] Wilcoxon's signed-rank test was used to test differences in the log IF values of each bacterial group. *, $P < 0.05$ for FV1 versus SV; **, $P < 0.005$ for FV1 versus SV; #, $P < 0.05$ for FV2 versus SV.

are qualitative analyses of vaginal communities, in terms of the presence or absence of the targeted bacterial groups, or relative quantitative evaluations in comparative studies.

The set of probe pairs of the VaginArray provided good coverage of the major *Lactobacillus* species that normally inhabit the healthy vagina, as well as of the most reported opportunistic bacteria, particularly species or genera associated with the altered ecosystem typical of BV. *G. vaginalis* was not included among the bacterial targets of the VaginArray because of a typical bias of several 16S rRNA gene PCR-based approaches (37, 38). This bias consists in the low efficiency of amplification of members of the family *Bifidobacteriaceae*, which includes *G. vaginalis* (21, 39). Certainly, the lack of a probe for *G. vaginalis* is a limit of the VaginArray, as this bacterium plays a key role in the vaginal niche, particularly in the case of BV. Therefore, the VaginArray must be implemented with a qPCR protocol designed to specifically quantify *G. vaginalis* (18, 40) in order to get a more comprehensive picture of the vaginal ecosystem, as proposed by Centanni et al. (41). The VaginArray recognized without ambiguity the 16S rRNA amplicons obtained from the principal members of the vaginal microbiota, confirming the specificity of all the probe pairs. PCR products were specifically identified with a detection limit of 6 to 12 ng of PCR product, demonstrating sensitivity comparable to that of other phylogenetic microarrays designed for the study of complex ecosystems (21).

The validation model for the VaginArray was a clinical study designed to assess the efficacy of rifaximin vaginal tablets for the treatment of BV (17). The clinical cure rate (80.0% [Amsel's criteria]) achieved with 25 mg/5 days rifaximin was similar to those reported for metronidazole and clindamycin (17). These current standard antibiotic therapies have clinical cure rates of 60% to 90% at 1 month; however, relief is often short-lived, and recurrence occurs in 15% to 30% of women within 1 to 3 months and 50% to 70% of women within 6 to 12 months (14).

In particular, the tool was applied to investigate the variations induced by rifaximin in the vaginal microbiota of 22 European patients. In general, the microarray data confirm the qPCR results related to the same clinical study described by Cruciani et al. (18). Both sets of data show an increase in the levels of members of the genus *Lactobacillus* and a decrease in the levels of BV-related bacteria after rifaximin treatment. This comparison, together with the correlations of microarray-based identification of lactobacilli and BV-related bacteria with Nugent scoring and Amsel's criteria, provides a demonstration of the VaginArray reliability in the correct characterization of the vaginal bacterial population. Hierarchical clustering on the IF data separated the BV-positive and BV-negative vaginal samples, with *A. vaginae* and *Bacteroides*/*Prevotella*, *Megasphaera*, and *Sneathia* species as the most represented bacterial groups in BV-positive samples. The antibiotic treatment reduced both the vaginal concentration and the prevalence of these BV-related bacterial groups. *A. vaginae* and *Sneathia* species showed the highest susceptibility to rifaximin, as indicated by the low prevalence at the first follow-up visit and the maintenance of the effect up to a month after antibiotic therapy. The population of lactobacilli was not compromised. Indeed, *L. iners* was the predominant species among lactobacilli at the baseline in all the recruited women and its prevalence remained almost unchanged throughout the study, confirming a previous observation of the absence of a correlation between *L. iners* presence and the onset of BV (13). More interestingly, the health-associated species *L. crispatus* and *L. vaginalis* showed a tendency to increase in prevalence, as well as in vaginal concentration in the case of *L. crispatus*, during the study period. These data indicated that rifaximin not only preserves the health-promoting autochthonous lactobacilli but may also nurture their proliferation, probably taking advantage of the disappearance of BV-related bacteria (18). In particular, the role of *L. crispatus* in the healing process of bacterial vaginosis was highlighted, opening the perspective of using probiotics containing this species as adjuvant therapeutic agents for the cure of BV.

In conclusion, the present work describes the development and validation of the VaginArray, a new phylogenetic microarray-based tool useful to quickly and efficiently study the fluctuations of the vaginal bacterial communities. The applicability of the tool in clinical trials was demonstrated by taking as a model the treatment of BV with rifaximin. VaginArray provides a comprehensive view of the health status of the vaginal ecosystem, which could be useful at a diagnostic level. Further, it enables recognition of which BV conditions could be effectively treated by the use of rifaximin, such as those characterized by a high abundance of *A. vaginae* and/or *Sneathia* spp. Finally, the results presented here also highlighted new aspects on the activity of rifaximin in the vagina, i.e., the ability to nurture the proliferation of some *Lactobacillus* species, opening new perspectives for the treatment of BV.

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