Efficacy of Rifaximin Vaginal Tablets in Treatment of Bacterial Vaginosis: a Molecular Characterization of the Vaginal Microbiota

Federica Cruciani,^a Patrizia Brigidi,^a Fiorella Calanni,^b Vittoria Lauro,^b Raffaella Tacchi,^b Gilbert Donders,^c Klaus Peters,^d Secondo Guaschino,^e and Beatrice Vitali^a

Department of Pharmaceutical Sciences, University of Bologna, Bologna, Italy^a; Alfa Wassermann S.p.A., Bologna, Italy^b; Department of Obstetrics and Gynaecology, General Hospital Heilig Hart Tienen and University Hospital Gasthuisberg, Catholic University of Leuven, Leuven, Belgium^c; Berner Heerweg 157, Hamburg, Germany^d; and Department of Obstetrics and Gynecology, Institute of Child Health, IRCCS Burlo Garofolo, Trieste, Italy^e

Bacterial vaginosis (BV) is a common vaginal disorder characterized by an alteration of the vaginal bacterial morphotypes, associated with sexually transmitted infections and adverse pregnancy outcomes. The purpose of the present study was to evaluate the impact of different doses of rifaximin vaginal tablets (100 mg/day for 5 days, 25 mg/day for 5 days, and 100 mg/day for 2 days) on the vaginal microbiota of 102 European patients with BV enrolled in a multicenter, double-blind, randomized, placebo-controlled study. An integrated molecular approach based on quantitative PCR (qPCR) and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) was used to investigate the effects of vaginal tablets containing the antibiotic. An increase in members of the genus *Lactobacillus* and a decrease in the BV-related bacterial groups after the antibiotic treatment were demonstrated by qPCR. PCR-DGGE profiles confirmed the capability of rifaximin to modulate the composition of the vaginal microbial communities and to reduce their complexity. This molecular analysis supported the clinical observation that rifaximin at 25 mg/day for 5 days represents an effective treatment to be used in future pivotal studies for the treatment of BV.

The homeostasis of the vaginal ecosystem results from complex interactions and synergies among the host and different microorganisms that colonize the vaginal mucosa (24). In healthy women, the vaginal ecosystem is dominated by lactobacilli, but a diverse array of other bacteria can be present in much lower numbers. Lactobacilli are involved in maintaining the normal vaginal microbiota by preventing overgrowth of pathogenic and opportunistic organisms (16, 34). The advent of culture-independent molecular approaches based on sequencing of 16S rRNA genes has advanced the understanding of the vaginal microbiota by identifying taxa that had not been cultured (17, 18, 31).

Bacterial vaginosis (BV), a common vaginal syndrome affecting pre- and postmenopausal, nonpregnant, and pregnant women, is a complex polymicrobial disorder associated with an increase in the taxonomic richness and diversity of the vaginal microbiota (20, 29). It is characterized by an overgrowth of *Gardnerella vaginalis*, anaerobic Gram-negative rods, anaerobic Gram-positive cocci, *Mycoplasma hominis*, and *Mobiluncus* spp. and a reduction in lactobacilli, particularly those producing hydrogen peroxide (20, 21, 40). The massive overgrowth of vaginal anaerobes is associated with increased production of proteolytic carboxylase enzymes, which act to break down vaginal peptides to a variety of amines (putrescine, cadaverine, and trimethylamine) which, at high pH, become volatile and malodorous. The amines are associated with increased vaginal transudation and squamous epithelial cell exfoliation, creating the typical discharge (6, 37). The clinical consequences of BV could be important. Indeed, 40% of cases of spontaneous preterm labor and preterm birth are thought to be associated with BV or other types of lactobacillus-devoid flora (9, 30). Furthermore, in this state of diminished colonization resistance, BV renders women particularly vulnerable to the acquisition of HIV and other sexually transmitted pathogens, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and herpes simplex virus type 2 infection (7, 25, 44). The diagnosis of BV is based on Amsel's criteria (1) or on Nugent score determinations

(28). Current therapy for BV involves effective oral or intravaginal administration of metronidazole or clindamycin, but long-term follow-up suggests recurrence rates as high as 58% 1 year after treatment (4). The reasons for recurrence may include the failure to eradicate the offending organisms, due to the formation of a prolific bacterial biofilm adhering to the vaginal epithelium (38). The main components of this polymicrobial biofilm are *G. vaginalis* and *Atopobium vaginae*. Cultivation-independent analysis of BV microbiota following metronidazole treatment confirmed the incapability of this antibiotic to reduce the bacterial levels in subjects with persistent BV (12). The high recurrence rates, which result in repeated exposure to antibiotics and the emergence of drug-resistant strains, suggest a need for alternative therapeutic tools.

Rifaximin is a semisynthetic rifamycin derivative with a broad antimicrobial spectrum (33, 35) and a good safety profile because of its negligible grade of systemic absorption. Thanks to its antibacterial activity, which covers *G. vaginalis* and other pathogens responsible for urogenital infections (15), rifaximin could be a suitable alternative for the local treatment of BV.

The aim of the present study was to evaluate the impact of two doses of rifaximin vaginal tablets (100 mg and 25 mg) administered for different periods (2 days and 5 days) on the vaginal microbiota of women affected by BV. Bacterial DNA extracted from standardized vaginal rinse fluids was analyzed using the culture-

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Address correspondence to Beatrice Vitali, b.vitali@unibo.it.

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independent techniques PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and real-time quantitative PCR (qPCR). PCR-DGGE represents a rapid and reliable technique used to examine microbial diversity and community structure in specific ecological niches. It has been widely applied for comparative analysis of parallel samples and can be used to monitor the shifts of the dominant bacterial taxa which determine the onset of a vaginal infection, such as BV (20, 21, 41). qPCR with genus- and species-specific primers provides a quantitative approach targeted against specific bacterial groups present in complex microbial communities, allowing the monitoring of changes in the amounts of specific bacterial taxa in response to an infectious state or to an antibiotic treatment (3, 12, 41). These techniques help to define the potential pathogenic populations in BV and provide new insights into the etiology and treatment of the disease.

MATERIALS AND METHODS

Study population. A multicenter, double-blind, randomized, placebo-controlled study (EudraCT: 2009-011826-32) was performed to compare the efficacy of rifaximin vaginal tablets versus placebo for the treatment of BV. A total of 102 Caucasian, premenopausal, nonpregnant women, 18 to 50 years old, from three European countries (Italy, Germany, and Belgium) were evaluated for the present molecular study. Diagnosis of BV was made using both Amsel's criteria and Nugent scoring at the screening visit (V1). Patients with a Nugent score of >3 and positive for at least three of four of Amsel's criteria were included. At the randomization visit (V2), the patients were distributed into 4 treatment groups: group A received a 100-mg rifaximin vaginal tablet once daily for 5 days ($n = 27$), group B received a 25-mg rifaximin vaginal tablet once daily for 5 days ($n = 25$), group C received a 100-mg rifaximin vaginal tablet once daily for the first 2 days and a placebo vaginal tablet for the remaining 3 days ($n = 25$), and group D received a placebo vaginal tablet once daily for 5 days ($n = 25$). Study medication was administered intravaginally at bedtime. At 7 days after the end of the therapy the first follow-up visit (V3) was performed. Patients showing remission according to Amsel's criteria (positive for ≤ 2) and Gram stain Nugent score (Nugent score ≤ 3) attended the second follow-up visit (V4) 28 days after the end of the treatment. Rifaximin and placebo vaginal tablets were purchased by Alfa Wassermann S.p.A., Bologna, Italy.

Standardized vaginal rinses with 2 ml of saline were collected for molecular studies at V1, V3, and V4 by flushing and reaspirating the fluid through a 22-gauge needle in the left, central, and right upper vaginal vaults as described elsewhere (8). The vaginal rinse fluids were subsequently stored at -80°C until use.

Extraction of bacterial DNA from vaginal rinse fluids. One ml of vaginal rinse fluids was centrifuged at $9,500 \times g$ for 15 min, and the pellets were washed three times in saline at 40°C . Each pellet was resuspended in 180 μl of enzymatic lysis buffer (20 mM Tris-HCl [pH 8], 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme) and incubated at 37°C for 30 min. Glass beads (200 mg) were added and the sample was mixed by vortexing for 1 min. Total DNA was extracted by using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) following the protocol "Pre-treatment for Gram-positive bacteria." A slight modification was introduced: a centrifugation step ($6,000 \times g$ for 5 min) was carried out after the incubation with proteinase K to remove glass beads. DNA amounts were quantified by using NanoDrop 1000 (Thermo Scientific, Wilmington, DE).

Real-time quantitative PCR. Quantitative PCR was performed in a LightCycler instrument (Roche, Mannheim, Germany), and SYBR green I fluorophore was used to correlate the amount of PCR product with the fluorescence signal. Each DNA sample was amplified with different genus- or species-specific primer sets targeted to 16S rRNA gene or 16S-23S rRNA spacer region: Bact-0011f/Lab-0677r (13) for *Lactobacillus*, F-GV1/R-GV3 (45) for *G. vaginalis*, c-Atopo-f/c-Atopo-r (27) for *Atopobium*, g-Prevo-f/g-Prevo-r (26) for *Prevotella*, VeilloF/VeilloR (32) for *Veillo-*

nella, MycF/MycR (46) for *M. hominis*, and Mob-s/Mob-as (39) for *Mobiluncus*. 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 either 2 μl of template or water (no-template control). The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 min followed by 30 (*Lactobacillus*, *Atopobium*, *G. vaginalis*, *Veillonella*, *Mobiluncus*), 35 (*Prevotella*), or 40 (*M. hominis*) cycles of denaturation at 95°C for 15 s; primer annealing at 63°C (*Lactobacillus*, *Mobiluncus*), 62°C (*Veillonella*, *M. hominis*), or 60°C (*Atopobium*, *Prevotella*, *G. vaginalis*) for 20 s; extension at 72°C for 45 s (*Lactobacillus*, *Atopobium*, *Prevotella*, *G. vaginalis*, *Veillonella*) or 30 s (*M. hominis*, *Mobiluncus*); and a fluorescence acquisition step at 82°C (*M. hominis*), 85°C (*Lactobacillus*, *Atopobium*, *G. vaginalis*, *Veillonella*), 87°C (*Prevotella*), or 88°C (*Mobiluncus*) for 5 s. DNAs extracted from *Lactobacillus acidophilus* NCFM, *Gardnerella vaginalis* ATCC 14018, *Prevotella bivia* ATCC 29303, *Veillonella parvula* ATCC 10790, *Atopobium vaginae* ATCC BAA-55, *Mycoplasma hominis* DSM 19104, and *Mobiluncus curtisii* ATCC 43063 were used as standards for PCR quantification. DNAs extracted from vaginal samples were amplified in triplicate for each primer set. Data were expressed as ng of DNA of the targeted genus or species per μg of total DNA extracted from the vaginal sample.

PCR-DGGE analysis. Amplification of the V2-V3 region of the bacterial 16S rRNA gene was carried out using the universal eubacterial primers GCclamp-HDA1 and HDA2 (43), supplied by M-Medical, Milan, Italy. The amplification reactions were performed in a Biometra T gradient thermal cycler (Biometra, Göttingen, Germany). GoTaq Flexi DNA polymerase (Promega, Madison, WI) was used as thermostable DNA polymerase. The reaction mixture contained each primer at 0.5 μM , each deoxynucleoside triphosphate (dNTP) at 200 μM , 2 mM MgCl_2 solution, 1.25 U of GoTaq Flexi DNA polymerase, 5 μl of 5 $\times$ Green GoTaq Flexi buffer, and 30 ng of the bacterial DNA template in a final volume of 25 μl . The thermocycler program consisted of the following time and temperature profile: 95°C for 5 min; 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 60 s; and 72°C for 8 min. Samples (5 μl) of the amplified products (200 bp) were subjected to gel electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining.

DGGE analysis was performed using a D-Code universal mutation system apparatus (Bio-Rad, Hercules, CA) with 20-cm by 20-cm by 0.75-mm gels. The sequence-specific separation of the PCR fragments was obtained in 8% (wt/vol) polyacrylamide (acrylamide-*N,N'*bisacrylamide, 40:3 [wt/vol]) gels in $0.5\times$ TAE buffer (20 mM Tris, 10 mM glacial acetic acid, and 0.5 mM EDTA [pH 8]). The denaturing gels contained a 30%-to-50% gradient of urea and formamide increasing in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7 M urea. A stacking gel containing 8% (wt/vol) polyacrylamide was applied to the denaturing gel. A volume of 8 μl of PCR samples was loaded on the stacking gel. Electrophoresis was conducted at an initial voltage of 150 V for 5 min and then at a constant voltage of 90 V and temperature of 60°C for 16 h. Following electrophoresis, the gel was silver stained (2) and scanned using a Gel Doc XR molecular imager system (Bio-Rad). Cluster analysis of the DGGE patterns was performed using the FPQuest software, version 4.5 (Bio-Rad), with the Ward clustering algorithm. The similarity indexes (SIs) of profiles were calculated on the basis of the Pearson correlation coefficient. The SI indicates the percent similarity among two or more DGGE profiles belonging to the same woman, taking into account the presence or absence and the intensity of the bands. The richness indexes (RIs) of the profiles were also calculated from the densitometric curves, using FPQuest. RI indicates the number of bands of each DGGE profile and reflects the microbial complexity of the ecosystem. SI and RI are parameters which describe how the bacterial composition changes following a stimulation or a perturbation of the ecological niche.

Statistical analysis. Statistical analysis was performed using SigmaStat (Systat Software, Point Richmond, CA). Differences in the amounts of the bacterial genera and species determined by qPCR were analyzed using

TABLE 1 Geographic origins and rates of remission

Treatment group ^a	No. of women	No. of women from:			No. (%) in remission at V3
		Italy	Germany	Belgium	
A	27	7	9	11	6 (22)
B	25	2	11	12	12 (48)
C	25	1	13	11	9 (36)
D	25	2	12	11	4 (16)

^a A, 100 mg/day for 5 days; B, 25 mg/day for 5 days; C, 100 mg/day for 2 days; and D, placebo.

Wilcoxon’s signed rank test. Kruskal-Wallis one-way analysis of variance (ANOVA) on the rank test was applied to compare the SIs of the DGGE profiles related to V1-V3 for all women and to V1-V3 and V3-V4 for women who were in remission at V3. Subsequently, Dunn’s test was used to make all pairwise comparisons. Differences in the SIs for different dosages and duration of treatment were evaluated by a two-way ANOVA test. Wilcoxon’s signed rank test was used to compare the RIs of the DGGE profiles related to V1-V3 for all women and to V1-V3 and V3-V4 for women who were in remission at V3. Finally, a Friedman repeated-measures ANOVA on the rank test was used to compare the RIs of the DGGE profiles related to V1, V3, and V4 for women who were in remission at V3, and Tukey’s test was then used to make all pairwise comparisons. A *P* value below 0.05 was considered significant in all statistical tests.

RESULTS

Clinical results. Remission was evaluated at the first follow-up visit (V3) by the combined assessment of Amsel’s criteria (clinical cure) and the Gram stain Nugent scoring system. Table 1 shows the geographic origin of the study population and the rate of remission for the treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days), and D (placebo). The highest percentage of remission was found for group B (48%), while lower percentages were calculated for the other treated groups (A, 22%; C, 36%). The lowest rate of remission was found for the group treated with placebo vaginal tablets (D, 16%). These clinical outcomes are reported in more detail in a separate paper (G. G. Donders, S. Guaschino, K. Peters, R. Tacchi, and V. Lauro, submitted for publication).

Variations in lactobacilli and BV-related bacterial populations. qPCR was performed to quantify *Lactobacillus*, *G. vaginalis*, *Atopobium*, *Prevotella*, *Veillonella*, *M. hominis*, and *Mobiluncus*, which are the principal bacterial groups that change in the presence of BV (3, 18, 41). The efficiency of qPCR for all assays was between 90% and 110%, and correlation coefficients for genomic DNA standards were >0.99.

At the baseline (V1), the percentages of women harboring *Lactobacillus*, *G. vaginalis*, *Atopobium*, *Prevotella*, *Veillonella*, *M. hominis*, and *Mobiluncus* were 95%, 89%, 99%, 98%, 30%, 78%, and 20%, respectively. These data show the high frequency of occurrence of *G. vaginalis*, *Atopobium*, *Prevotella*, and *M. hominis* in the vaginal ecosystem of women affected by BV, differently from *Veillonella* and *Mobiluncus*, which were detected in less than 50% of the patients.

Quantitative variations of lactobacilli and BV-related bacterial groups were visualized in scatter plots (Fig. 1 and 2) showing the distributions of qPCR data expressed as log nanograms of DNA of the target genus or species per microgram of total bacterial DNA extracted from the vaginal sample, with the horizontal bars representing the log median values.

Figure 1 shows the qPCR results at V1 and V3 related to all the

women analyzed (*n* = 102), randomized into the four treatment groups (A, B, C, and D). Treatment with 100 mg/day rifaximin for 5 days (group A) induced a slight increase in the amount of *Lactobacillus* and a decrease in *G. vaginalis* and *Atopobium*, with a significant reduction only for *Atopobium* [V1, 1.73 log ng/μg; V3, 0.93 log ng/μg (*P* = 0.02)]. At V3, the log median value of this genus was similar to that of lactobacilli. Concentrations of the other BV-related groups did not change after the treatment. In response to the administration of rifaximin 25 mg/day for 5 days (group B), *Lactobacillus* significantly increased [V1, 0.75 log ng/μg; V3, 1.28 log ng/μg (*P* = 0.03)] and *G. vaginalis* [V1, 1.67 log ng/μg; V3, −3.40 log ng/μg (*P* = 0.01)], *Atopobium* [V1, 1.96 log ng/μg; V3, 0.64 log ng/μg (*P* = 0.005)] and *Prevotella* [V1, 0.72 log ng/μg; V3, −0.99 log ng/μg (*P* = 0.004)] significantly decreased at V3. A slight decrease at V3 was also observed for *M. hominis*. Of all bacterial concentrations at V3, the log median value for *Lactobacillus* was the highest. These data indicated the efficacy of 25 mg/day rifaximin for 5 days in reducing BV-related bacteria and restoring lactobacilli and are in strong agreement with the high rate of remission (48%) assessed by Amsel’s criteria together with the Nugent scoring system. Treatment with 100 mg/day rifaximin for 2 days (group C) resulted in a significant reduction of *G. vaginalis* [V1, 1.65 log ng/μg; V3, 1.16 log ng/μg (*P* = 0.01)], *Atopobium* [V1, 2.12 log ng/μg; V3, 1.11 log ng/μg (*P* = 0.003)], *Prevotella* [V1, 1.18 log ng/μg; V3, −0.52 log ng/μg (*P* = 0.003)] and *M. hominis* [V1, −1.13 log ng/μg; V3, −2.12 log ng/μg (*P* = 0.01)], together with a slight reduction of *Lactobacillus* genus. Unlike in group B, in group C the log median values of *G. vaginalis* and *Atopobium* at V3 were higher than that of lactobacilli, suggesting that 100 mg/day rifaximin for 2 days was less effective against BV than 25 mg/day rifaximin for 5 days. In the placebo group (group D), a reduction in the amounts of all the bacterial groups was observed, with statistical significance for *Lactobacillus* [V1, 0.76 log ng/μg; V3, 0.38 log ng/μg (*P* = 0.007)], *G. vaginalis* [V1, 1.92 log ng/μg; V3, 1.55 log ng/μg (*P* = 0.04)] and *Atopobium* [V1, 1.83 log ng/μg; V3, 1.49 log ng/μg (*P* = 0.005)]. However, the log median values of *G. vaginalis* and *Atopobium* remained higher than that of lactobacilli at V3. In general, no variations were observed for *Veillonella* and *Mobiluncus*, as these genera were found in a low percentage of women, as described above, and at low concentrations (<−0.47 log ng/μg and <1.56 log ng/μg, respectively). However, a decrease in the percentage of women who harbored *Veillonella* and *Mobiluncus* was observed in all the treatment groups at V3 (*Veillonella*: A, V1, 11.1%, versus V3, 7.4%; B, V1, 24.0%, versus V3, 8.0%; C, V1, 28.0%, versus V3, 12.0%; D, V1, 28.0%, versus V3, 24.0%); *Mobiluncus* (A, V1, 4.0%, versus V3, 0%; B, V1, 8.0%, versus V3, 0%; C, V1, 20.0%, versus V3, 4.0%; D, V1, 16.0%, versus V3, 12.0%).

Figure 2 shows the qPCR results at V1, V3, and V4 related to the women who were in remission at V3 and who performed the last study visit (V4) (*n* = 31), divided into the four treatment groups. Treatment with 100 mg/day rifaximin for 5 days (group A) did not significantly affect any bacterial group, even though a reduction trend was observed for the BV-related bacteria, and *Lactobacillus* log median concentration after the treatment was higher than that of the other genera and species. Treatment with 25 mg/day rifaximin for 5 days (group B) caused an increase of *Lactobacillus* at V3 and V4, which was significant at V4 [V1, 0.72 log ng/μg; V4, 1.57 log ng/μg (*P* = 0.04)]. A significant decrease at V3 and V4 compared to V1 was observed for *G. vaginalis* [V1, 1.73

FIG 1 qPCR evaluation of *Lactobacillus*, *G. vaginalis*, *Atopobium*, *Prevotella*, *Veillonella*, *M. hominis*, and *Mobiluncus* at V1 and V3 for treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days), and D (placebo). Data are those for the total number of women analyzed ($n = 102$) and are expressed as log ng of DNA of the target genus per μg of total bacterial DNA extracted from the vaginal sample. The scatter plots show the distributions of quantification data, with the horizontal bars representing the median values. *, $P < 0.05$; **, $P < 0.01$.

log ng/ μg ; V3, -3.40 log ng/ μg ; V4, -3.40 log ng/ μg ($P = 0.004$, V3 versus V1) ($P = 0.004$, V4 versus V1)], *Atopobium* [V1, 2.02 log ng/ μg ; V3, 0.52 log ng/ μg ; V4, 0.56 log ng/ μg ($P = 0.004$, V3 versus V1) ($P = 0.02$, V4 versus V1)] and *M. hominis* [V1, -2.32 log ng/ μg ; V3, -2.81 log ng/ μg ; V4, -3.40 log ng/ μg ($P = 0.02$, V3 versus V1) ($P = 0.04$, V4 versus V1)]. *Prevotella* decreased at V3 and V4, with a significant variation at V3 only [V1, 0.95 log ng/ μg ; V3, -1.27 log ng/ μg ($P = 0.002$)]. Treatment with 100 mg/day rifaximin for 2 days (group C) showed a relapse at V4, with *Lactobacillus* concentration increasing at V3 and significantly decreasing at V4 compared to V3 [V3, 1.80 log ng/ μg ; V4, 0.33 log ng/ μg ($P = 0.03$)], and BV-related bacterial groups decreasing at V3 and increasing again at V4, with significance for *G. vaginalis* [V1, 1.91 log ng/ μg ; V3, -3.40 log ng/ μg ; V4, 1.96 log ng/ μg ($P = 0.005$, V3 versus V1) ($P = 0.05$, V4 versus V3)], *Atopobium* [V1,

2.09 log ng/ μg ; V3, 0.98 log ng/ μg ; V4, 2.17 log ng/ μg ($P = 0.003$, V3 versus V1) ($P = 0.005$, V4 versus V3)] and *Prevotella* [V1, 1.18 log ng/ μg ; V3, -1.06 log ng/ μg ; V4, 1.79 log ng/ μg ($P = 0.008$, V3 versus V1) ($P = 0.03$, V4 versus V3)]. Despite the absence of significant variations in the placebo group (group D), *Lactobacillus* gradually decreased at V3 and V4 while *Prevotella* decreased at V3 and increased again at V4. Notably, the *Lactobacillus* log median concentration was lower than that of *G. vaginalis* and *Atopobium* after treatment. Similarly to the analysis of V1 and V3 for the total number of women, no variations were observed for *Veillonella* and *Mobiluncus*, due to the low concentrations (< -0.77 log ng/ μg and < 1.01 log ng/ μg , respectively) and the low frequency of occurrence of these genera.

Bacterial population profiling with PCR-DGGE. PCR-DGGE analysis with universal primers for bacteria (HDA1-GC/HDA2)

FIG 2 qPCR analysis of *Lactobacillus*, *G. vaginalis*, *Atopobium*, *Prevotella*, *Veillonella*, *M. hominis*, and *Mobiluncus* at V1, V3, and V4 for treatment groups A (100 mg/day for 5 days), B (25 mg/day for 5 days), C (100 mg/day for 2 days), and D (placebo). Data are those for women who were in remission at V3 and performed the last study visit (V4) ($n = 31$). Data are expressed as log ng of DNA of the target genus per μg of total bacterial DNA extracted from the vaginal sample. The scatter plots show the distributions of quantification data, with the horizontal bars representing the median values. *, $P < 0.05$, and **, $P < 0.01$, relative to V1; °, $P < 0.05$, and °°, $P < 0.01$, relative to V3.

was carried out on the vaginal samples collected at V1, V3, and V4 to evaluate the impact of rifaximin on the composition of BV microbiota.

Cluster analyses were performed on DGGE profiles grouped on the basis of the treatment. Figure 3 represents the cluster for the treatment group B (25 mg/day for 5 days), while clusters for groups A (100 mg/day for 5 days), C (100 mg/day for 2 days) and D (placebo) are reported in the supplemental material. The percentages of women with V1 and V3 profiles clustering together were 37%, 24%, 28%, and 56% for the treatment groups A, B, C, and D, respectively. This result highlights the ability of rifaximin to modulate the vaginal microbiota composition exerting the strongest impact at the dosage of 25 mg/day for 5 days. By considering the women who were in remission at V3 and performed the

last study visit (V4), the percentages of V1, V3, and V4 DGGE profiles clustering together were 0% for treatment groups A and B, 11% for group C, and 50% for group D, further confirming the impact of rifaximin on the vaginal communities.

Table 2 reports the mean similarity indexes (SIs) of the DGGE profiles related to V1-V3 for all women analyzed and to V1-V3 and V3-V4 for the women in remission, according to the different treatment groups. Considering the total number of women, the highest mean SI for V1-V3 was found for group D, which received placebo (75.4%). Among the groups given rifaximin, the highest SI mean value was found for the treatment group A (61.6%), followed by C (54.0%) and B (48.4%). Considering the women who were in remission at V3, a similar trend was observed for SI mean values of V1-V3. The placebo group presented the high-

FIG 3 Cluster of DGGE profiles of vaginal samples collected from women belonging to treatment group B (25 mg/day for 5 days). ID, patient identification number; V, visit number; G, treatment group. Similarity values are indicated at each node.

TABLE 2 Mean SIs of DGGE profiles

Treatment group	Mean SI (%) for:		
	All women ($n = 102$), V1–V3	Women in remission ($n = 31$)	
		V1–V3	V3–V4
A	61.6	53.3	66.8
B	48.4	45.3	62.1
C	54.0	40.4	42.8
D	75.4	83.9	58.7

est value (83.9%), followed by the treatment groups A (53.3%), B (45.3%) and C (40.4%). The lower SI mean values of V1–V3 for the groups given rifaximin compared to the placebo group support the previously formulated hypothesis that the antibiotic treatment induces changes in the structure of BV microbiota. By considering the SI mean values of V3–V4 for the women in remission, a maintenance of the antibiotic effect on the vaginal communities was deduced for the treatment groups A and B, on the basis of their high SIs (66.8% and 62.1%, respectively). On the contrary, the SI mean value for group C was the lowest one (42.8%), indicating that the changes induced at V3

TABLE 3 Mean RIs of DGGE profiles

Treatment group	Mean RI for:				
	All women (<i>n</i> = 102)		Women in remission (<i>n</i> = 31)		
	V1	V3	V1	V3	V4
A	13.5	10.9	12.5	9.8	10.2
B	14.0	10.7	13.6	8.2	9.0
C	15.4	12.2	16.2	10.4	14.6
D	13.3	13.9	11.3	10.8	9.5

by rifaximin 100 mg/day for 2 days were not preserved until the last time point V4.

SIs were compared using the Kruskal-Wallis one-way ANOVA on the rank test. SIs of V1-V3 related to the total number of women were significantly different when all treatment groups were compared ($P < 0.001$). In particular, Dunn's test revealed significant differences for A versus D, B versus D, and C versus D ($P < 0.05$). Also, for the women in remission at V3, SIs of V1-V3 were significantly different when all treatment groups were compared ($P < 0.05$); a pairwise comparison by Dunn's test revealed significant differences for B versus D and C versus D ($P < 0.05$).

A two-way ANOVA was used to compare the influence of the two variables, dosage (100 mg versus 25 mg) and duration (5 days versus 2 days) of treatment, on SIs of DGGE profiles related to V1-V3. A significant difference was found for women who received different doses of antibiotic ($P < 0.05$), while no difference was observed for women treated for a different number of days ($P > 0.05$). This result indicates that the dosage exerts the major influence on the vaginal microbiota composition.

Table 3 shows the mean richness indexes (RIs) for DGGE profiles related to V1 and V3 for all women and to V1, V3, and V4 for the women who were in remission at V3, according to each treatment group. Considering the total number of women, a reduction of the mean RIs at V3 was observed in the groups given rifaximin, A (V1, 13.5, versus V3, 10.9), B (V1, 14.0, versus V3, 10.7), and C (V1, 15.4, versus V3, 12.2). Similar mean RIs at V1 and V3 were found for the placebo group (V1, 13.3, versus V3, 13.9). The mean RIs for the women in remission decreased at V3 compared to V1 in all groups treated with the antibiotic (A, V1, 12.5, versus V3, 9.8; B, V1, 13.6, versus V3, 8.2; C, V1, 16.2, versus V3, 10.4). The general decrease of RI at V3 indicates the capability of the antibiotic to reduce the complexity of the vaginal microbiota, complexity that is a typical feature of BV. Mean RIs at V4 were similar to those calculated at V3 for groups A (10.2) and B (9.0), indicating a maintenance of the antibiotic effect. In contrast, the mean RI for group C (14.6), after the decrease at V3, approximated the baseline value at V4, reflecting a restoration of the complexity of the BV microbiota.

Wilcoxon's signed rank test revealed significant differences in RIs between V1 and V3 for groups A ($P < 0.05$), B ($P < 0.05$), and C ($P < 0.001$), considering the total number of women, and for groups B ($P < 0.001$) and C ($P < 0.001$), considering the women in remission. Friedman repeated measures ANOVA on the rank test showed a significant difference of RIs among V1, V3, and V4 in groups B ($P < 0.05$) and C ($P < 0.001$); a further pairwise analysis with Tukey's test revealed significant differences for V1 versus V3 and V1 versus V4 in group B and for V1 versus V3 and V3 versus V4 in group C ($P < 0.05$). These results stress again the

efficacy of 25 mg/day rifaximin for 5 days in the follow-up period and the risk of relapse associated with 100 mg/day rifaximin for 2 days.

In conclusion, DGGE results are in strong agreement with qPCR data, confirming and reinforcing them. This integrated molecular analysis allowed us to identify the best treatment regimen for the cure of BV, which is 25 mg/day rifaximin for 5 days.

DISCUSSION

Symbiosis between the vaginal microbiota and the human host is crucial for reproductive tract health. The importance of this relationship is reflected by the fact that BV has serious gynecological and obstetric complications, and it creates a permissive environment for acquiring sexually transmitted infections (40). Conventional treatments of BV are represented by metronidazole and clindamycin, but their effectiveness seems to be limited, as evidenced by a BV recurrence rate of 58% within 6 months. The high recurrence rate could be interpreted as an incomplete eradication of BV-related bacteria after antibiotic treatment, though a new infection could also be possible (4). Moreover, these treatments play a significant role in the expansion of drug resistance in *G. vaginalis* and *Prevotella* spp. (5, 19). The failure of conventional antibiotics in BV cure and eradication raises the question of alternative therapeutic tools.

Rifaximin is a nonabsorbable broad-spectrum antibiotic that is associated with a low risk of bacterial resistance. While several studies report the efficacy of rifaximin in the treatment of gastrointestinal disorders (33, 35), the use of this antibiotic in genital tract infections has not been explored until now. The purpose of the present study was to evaluate the efficacy of different doses of rifaximin vaginal tablets (100 mg/day for 5 days, 25 mg/day for 5 days, 100 mg/day for 2 days) in BV cure and remission maintenance of 102 European patients. A combination of two molecular techniques, PCR-DGGE and qPCR, was used to achieve a detailed overview of the bacterial composition of the vaginal environment in relation to the BV condition and to the effects of the antibiotic regimen.

Consistent with data from previous studies (12, 20), our qPCR results showed that BV-associated microbiota is dominated by several anaerobic bacterial species which coexist with lactobacilli. These anaerobic bacteria are generally undetectable or found at very low concentrations (< 2 ng of target bacterial DNA per μ g of total DNA) in a healthy vagina but dramatically increase in women developing BV (3). At the screening visit (V1) of the present study, *Atopobium* and *G. vaginalis* were the predominant bacteria, found in larger amounts than lactobacilli. *Prevotella* showed a baseline concentration similar to that of *Lactobacillus*, while *Veillonella* and *Mobiluncus*, which have previously been associated with BV infection (3, 14, 41), were detected in only a few women and in small amounts. *M. hominis* was recovered from 78% of BV-positive women, while *Mobiluncus* was present only in 20%. According to the morphology, as determined via Gram stains, *Mobiluncus* was suspected in 26% of cases, as their Nugent score was 9 or 10. The mismatch between microscopic findings and the molecular data could be a function of the presence of BV-associated bacteria belonging to the order *Clostridiales* (BVAB1), whose Gram stain shows a morphotype similar to that of *Mobiluncus* (11). In a previous study (23), the concentration of *A. vaginae* was found to correlate extremely well with the Nugent scores, with the highest *A. vaginae* concentration being found in patients with Nu-

gent scores of 9 and 10. This leads us to the suggestion that *Atopobium* should be seen as one of the most important markers of severity of the microbial disturbance encountered in BV. Furthermore, as *A. vaginae* was also recovered in smaller amounts in vaginal fluid with intermediate Nugent scores, i.e., 4 to 6, and *Mobiluncus* is represented only by a score of 9 or 10, the role of the latter seems to have been overrated in the past, and its importance in the diagnosis of BV should be reappraised.

By comparing the qPCR data from the follow-up visits (V3 and V4) with those from the screening visit (V1), we sought to determine whether changes in quantities of vaginal bacteria, as reflected by bacterial DNA concentrations, are associated with the cure of BV after rifaximin therapy. In particular, we aimed at identifying the most efficient treatment able to reduce the concentrations of BV-related bacteria and to allow *Lactobacillus* spp. to recolonize the vaginal environment, reaching the highest abundance in the community. We demonstrated that the treatment with the highest dosage of rifaximin (100 mg/day for 5 days, group A) was less effective, as only the levels of *Atopobium* organisms significantly decreased after the antibiotic intake, and lactobacilli did not succeed in recolonizing the vaginal mucosa. The intake of rifaximin 100 mg/day for 2 days (group C) gave a better response at the first follow-up visit (V3). All the BV-associated bacteria significantly decreased, in terms of either concentration (*Atopobium*, *G. vaginalis*, *Prevotella*, and *M. hominis*) or percentage of presence (*Veillonella* and *Mobiluncus*), while *Lactobacillus* levels significantly increased, reaching higher values than in all the other groups in the women who were in remission at V3. However, bacterial concentrations at the second follow-up visit (V4) were similar to the baseline, indicating a relapse that was probably due to the short duration of the antibiotic intake. Rifaximin at 25 mg/day for 5 days (group B) was found to be the most effective treatment, giving a good response at V3 and maintaining the effect until V4. Indeed, a significant increase in *Lactobacillus* organisms together with a significant decline of bacteria associated with BV was observed at both V3 and V4. The lack of *in vivo* rifaximin susceptibility of lactobacilli is not surprising. This peculiar feature had already been pointed by previous studies reporting that rifaximin did not affect the intestinal amounts of several health-promoting microbial groups, such as *Lactobacillus* spp., *Bifidobacterium* spp., and *Atopobium/Collinsella* spp. (22). Our hypothesis that rifaximin at 25 mg/day for 5 days (group B) represents the most effective treatment against BV is in accordance with the rates of remission at V3 assessed by both Amsel's and Nugent criteria. In fact, the highest percentage of remission was found for group B (48%), while lower percentages were calculated for the other treated groups (A, 22%; C, 36%). The lowest rate of remission was found for the group treated with placebo vaginal tablets (D, 16%), confirming that spontaneous remission at low rates is possible, as reported elsewhere (36, 42).

Information derived from qPCR assays related to specific targeted bacteria was integrated with the PCR-DGGE data, which provided an overall picture of the diversity and richness of the global bacterial microbiota. Cluster analyses of DGGE profiles revealed a higher intraindividual variability in the women treated with the antibiotic than in the women given the placebo, highlighting the ability of rifaximin to modulate the vaginal microbiota composition. This assumption was made by considering that treatment groups A, B, and C presented lower percentages of women with V1 and V3 profiles clustering together and lower

similarity indexes (SIs) for V1-V3 profiles than the placebo group. In particular, the lowest SIs were found for V1-V3 profiles of women belonging to groups B and C, when considering the total number of women and the number who were in remission at V3, respectively. Maintenance of the antibiotic effect on the vaginal bacterial communities was demonstrated for treatment groups A and B on the basis of their highest SIs of V3-V4 profiles related to the women who were in remission at V3. The lowest SI of group C suggested that the treatment with 100 mg/day rifaximin for 2 days did not avoid relapse. In general, these results confirm the hypothesis suggested by qPCR data that the treatment with rifaximin 25 mg/day for 5 days is the most effective. In fact, the low similarity of V1-V3 DGGE profiles of group B demonstrates that the antibiotic intake induced a variation in the overall composition of the vaginal microbiota, while the high similarity of V3-V4 DGGE profiles for the women in remission indicates the maintenance of the effect. The identification of rifaximin at 25 mg/day for 5 days as the best treatment for the cure of BV was further supported by the finding that the variable dosage exerted a major influence on the diversity of the vaginal microbiota compared to the variable duration of the treatment.

Previous PCR-DGGE studies indicated that the level of complexity of the vaginal microbiota was higher in individuals with BV infection than in healthy individuals (21, 41). Here, the complexity of the vaginal communities was evaluated by calculating the richness indexes (RIs) of DGGE profiles. The significant decrease of RI values after the antibiotic intake in all the treated groups indicates the rifaximin effectiveness in reducing the complexity of the vaginal ecology and restoring a community structure similar to a healthy microbiota. The RI values at V4 further confirmed the maintenance of remission in women treated with 25 mg/day rifaximin for 5 days and the relapse occurring in women treated with 100 mg/day rifaximin for 2 days.

In conclusion, our molecular results suggest that rifaximin is a good candidate for the treatment of BV. The perfect match with clinical outcomes highlights the power of molecular techniques to explore the mechanisms underlying the therapeutic effects. In particular, treatment with 25 mg/day rifaximin for 5 days is active against the pool of bacteria recognized as causing BV, while it does not affect the normal population of lactobacilli. A selective action against fastidious bacteria is a crucial feature of a successful therapy for BV. This feature has also been demonstrated by molecular analyses for vaginal metronidazole treatment (10, 12), even though this antibiotic has proved unable to reduce the concentrations of BV-associated bacteria in cases of persistent BV (12). Based on the encouraging results reported in the present work, further clinical studies will be undertaken in order to confirm the real advantage of rifaximin in comparison to standard BV treatments.

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