CHAPTER 5

The vaginal microbiota in the course of bacterial vaginosis treatment

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ABSTRACT

Bacterial vaginosis (BV) is perceived as a condition of disrupted vaginal microbiota, but remains of unknown aetiology. In this study, vaginal microbiota composition was determined in twenty-one women with BV, before and after treatment with metronidazole or clindamycin. Microbiota composition varied greatly between women and defining a (un) healthy vaginal microbiota state remains elusive, challenging BV diagnosis and treatment. While relative abundance of *Lactobacillus* increased after antibiotic treatment in two-third of women, its abundance was not associated with treatment outcome. Instead, remaining complaints of abnormal vaginal discharge were more common after metronidazole treatment and associated with increased relative abundance of *Ureaplasma*.

INTRODUCTION

The vaginal microbiota plays a crucial role in maintaining a healthy vaginal environment and perturbation of this system has been implicated in disturbed vaginal health and other negative outcomes (1, 2). The vaginal microbiota is dynamic and influenced by hormonal changes, sexual activity and hygiene (3). Various vaginal bacterial communities exist in healthy women, mostly dominated by *Lactobacillus* species, while some are being composed of anaerobes like *Atopobium* and *Prevotella* species (4). Nevertheless, the common perception of a healthy vaginal microbiota is one dominated by one or more *Lactobacillus* species. As such, the switch from a *Lactobacillus*-dominated microbiota to a more diverse microbiota, in combination with clinical symptoms, is considered as bacterial vaginosis or aerobic vaginitis, depending on colonisation by anaerobic or aerobic bacteria, respectively. Bacterial genera that are specifically associated with BV are, amongst others, *Gardnerella*, *Atopobium*, *Prevotella*, *Fusobacterium* and *Dialister* species (5). Despite these associations, the aetiology of BV is unknown, and diagnosis and treatment remain elusive. While a Gram-stain evaluation according to the Nugent criteria is considered the golden standard for BV diagnosis, it is not routinely applied in a clinical setting (6). Instead, BV diagnosis is commonly based on clinical signs and symptoms or Amsel criteria (7). Symptoms of BV can resolve without intervention, but metronidazole or clindamycin can be prescribed in case of persistence, even though recurrence is common (8, 9). In our study, vaginal microbiota composition of women with clinically diagnosed BV was determined before and after antibiotic treatment and related to clinical characteristics.

MATERIALS AND METHODS

Prospectively, vaginal secretions and clinical data were collected from 60 premenopausal women visiting the Gynaecology outpatient clinic of the Haaglanden Medical Centre (The Hague, The Netherlands) with complaints of abnormal vaginal discharge. Vaginal secretion was collected using the ESwab (Copan Diagnostics Inc, USA). BV diagnosis was based on clinical symptoms and signs, with malodorous discharge as major criterium for diagnosis of bacterial vaginosis, followed by culturing when clinical diagnosis based on symptoms alone was uncertain. Therapy was initiated according to routine hospital practice following the European guideline and consisted of 500 mg metronidazole taken orally twice a day for seven days, or, in case of pregnancy or lactating, 300 mg clindamycin taken orally twice a day for seven days (13). A follow-up visit was scheduled approximately four weeks after inclusion, during which vaginal swab and clinical data collection were repeated. Women who were clinically diagnosed with BV and attended the follow-up visit were selected for microbiota profiling (n = 21). Clinical data collection, Amsel criteria (vaginal pH, amine odour,

wet-mount microscopy), Nugent score, and *Gardnerella vaginalis* culturing, were performed for research purposes as previously described (14). Detailed subject characteristics are outlined in **Table 1**. The Declaration of Helsinki was the guiding principle for trial execution, and the study was approved by the local ethics board (METC Zuidwest Holland, The Hague, The Netherlands). All patients provided written informed consent before participation.

Table 1. Patient characteristics

		Before treatment	After treatment
Demographics	N	21	21
	Age (mean ± SD years)	32.5 ± 7.6	32.5 ± 7.6
	European	15	15
Antimicrobials	Clindamycin	-	11
	Metronidazole	-	10
	Clotrimazole	-	4
	Azithromycin	-	1
Symptomology	Abnormal discharge	21	9
	Malodorous discharge	20	4
	Increased discharge	13	5
	Yellow/green discharge	7	2
	Curdy discharge	2	2
	Thin white discharge	8	5
	Purulent discharge	1	0
	Vulvar erythema oedema	4	2
	Vulvar itching	9	3
	Vulvar irritation	6	3
	Cervical erythema	3	2
	Cervical bleeding	1	0
	Low abdominal pain	10	3
Diagnosis	Bacterial vaginosis	21	2
	Nugent score positive	12	5
	Amsel criteria positive	13	4
	Vaginal pH > 4.5	18	12
	Amine odour	16	8
	Clue cells	14	4
Other	Anticonception	6	6
	Vaginal shower gel	0	1
	Sexually active	20	20
	Pregnant	8	8
	Lactating	3	3

SD: standard deviation.

Vaginal bacterial microbiota was determined by 16S rRNA gene amplicon sequencing of the V3-V4 region using the Nextera XT, MiSeq Reagent Kits v2 500 cycles and a MiSeq desktop sequencer (Illumina, USA). Raw sequencing data are available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under study accession PRJNA524112. Read filtering, operational taxonomic unit (OTU)-picking and taxonomic assignment were performed using the NG-Tax 0.4 pipeline and the Silva_132_SSU Ref database (10). Statistical analysis and data visualisation were performed in R (v3.5.1) using the packages phyloseq (v1.26.1), vegan (v2.5-4), ggplot2 (v3.1.0), DESeq2 (v1.22.2) microbiome (v1.4.2) and DirichletMultinomial (v1.24.1). For differential abundance testing by DESeq2, the OTU-table was filtered for OTUs present in less than 25% of the samples to minimize zero-variance errors and spurious significance. Permutational multivariate analysis of variance was performed using the adonis function with 999 permutations and Bray-Curtis distances to determine associations between microbiota composition and clinical variables. The Dirichlet Multinomial Mixtures method, using the Laplace equation, was applied for community typing. In this approach samples are clustered based on microbiota profile similarity (11). Kruskal-Wallis followed by post-hoc Dunn's testing was performed to compare Shannon diversity indices between groups.

RESULTS AND DISCUSSION

Before antibiotic treatment, genera *Gardnerella*, *Atopobium*, *Prevotella*, *Lactobacillus* and *Dialister* constituted the core microbiota, and combined accounted for an average relative abundance of 71.9% (**Table 2**), but their abundance could vary greatly between subjects (**Figure 1a**). Two community types could be identified, one driven by *Gardnerella*, *Prevotella*, *Sneathia* and *Atopobium* (community type 1), and one driven by *Lactobacillus*, *Gardnerella* and *Atopobium* (community type 2, **Figure 2a**), suggesting *Lactobacillus*, *Prevotella* and *Sneathia* abundances as discriminative feature of microbiota composition between patients. Bacterial diversity significantly differed between the two community types (**Figure 3a**), with lower diversity in the *Lactobacillus* driven community type. Microbiota composition before treatment was significantly associated with various parameters (**Table 3**), including Nugent score, hormone-related variables (lactation, anticonception use) and BV symptomology (vaginal pH and amine odour).

After treatment, bacterial diversity was decreased (**Figure 3c**) and the core microbiota solely consisted of *Lactobacillus*, constituting an average of 60.8% relative abundance (**Table 2**). Independent of antibiotic type (metronidazole or clindamycin), antibiotic treatment significantly decreased the relative abundance of *Atopobium* (Log2FoldChange = -3.36, padj = 0.0388), while increasing *Lactobacillus* (Log2FoldChange = 4.04, padj = 0.0002). However, *Lactobacillus* remained of low abundance in one-third of the women,

Table 2. Core microbiota before and after antibiotic treatment. Bacterial taxa were considered part of the core microbiota when present in 75% of the samples from the specified group.

	Bacterial genus	Average relative abundance (fraction)
Before treatment	*Gardnerella*	0.294
	Atopobium	0.104
	Prevotella	0.132
	Lactobacillus	0.151
	Dialister	0.038
After treatment	*Lactobacillus*	0.608

Table 3. Clinical variables significantly associated with microbiota composition before and after antibiotic treatment

	Variable	R2	p-value
Before treatment	Nugent score	0.238	0.001
	Anticonception	0.146	0.008
	Lactating	0.091	0.008
	pH > 4.5	0.086	0.012
	Amine odour	0.073	0.028
After treatment	Nugent score	0.499	0.001
	pH > 4.5	0.143	0.006

who's microbiota was of individual-specific composition with high abundance of either *Gardnerella*, *Prevotella*, *Dialister*, *Escherichia-Shigella*, *Atopobium* or *Sneathia* (**Figure 1b**). These microbiota compositions were also reflected by the identification of two community types; one driven by *Lactobacillus*, and the other driven by multiple bacterial taxa (**Figure 2b**), with lower diversity in the *Lactobacillus*-driven community type (community type 1, **Figure 3b**). Vaginal microbiota composition after antibiotic treatment was significantly associated with Nugent score and vaginal pH (**Table 3**).

These findings support the current debate on the definition of a healthy vaginal microbiota (12), since *Lactobacillus* dominance was observed in a large proportion of women with symptoms and the opposite, dominance of anaerobes, was observed in asymptomatic women. So even in a study of small subject size, as herein, heterogeneity of vaginal bacterial communities was apparent. Vaginal health status may be associated with specific *Lactobacillus* species (13), which could not be defined by the method used herein. However, several kinds of microbiota composition existed in asymptomatic women, which has been previously reported (4, 14, 15). Vaginal microbiota composition was consistently associated with Nugent score and vaginal pH. While the Nugent score is considered the golden standard for BV diagnosis, it is rarely used in clinical setting due to resource intensiveness (6).

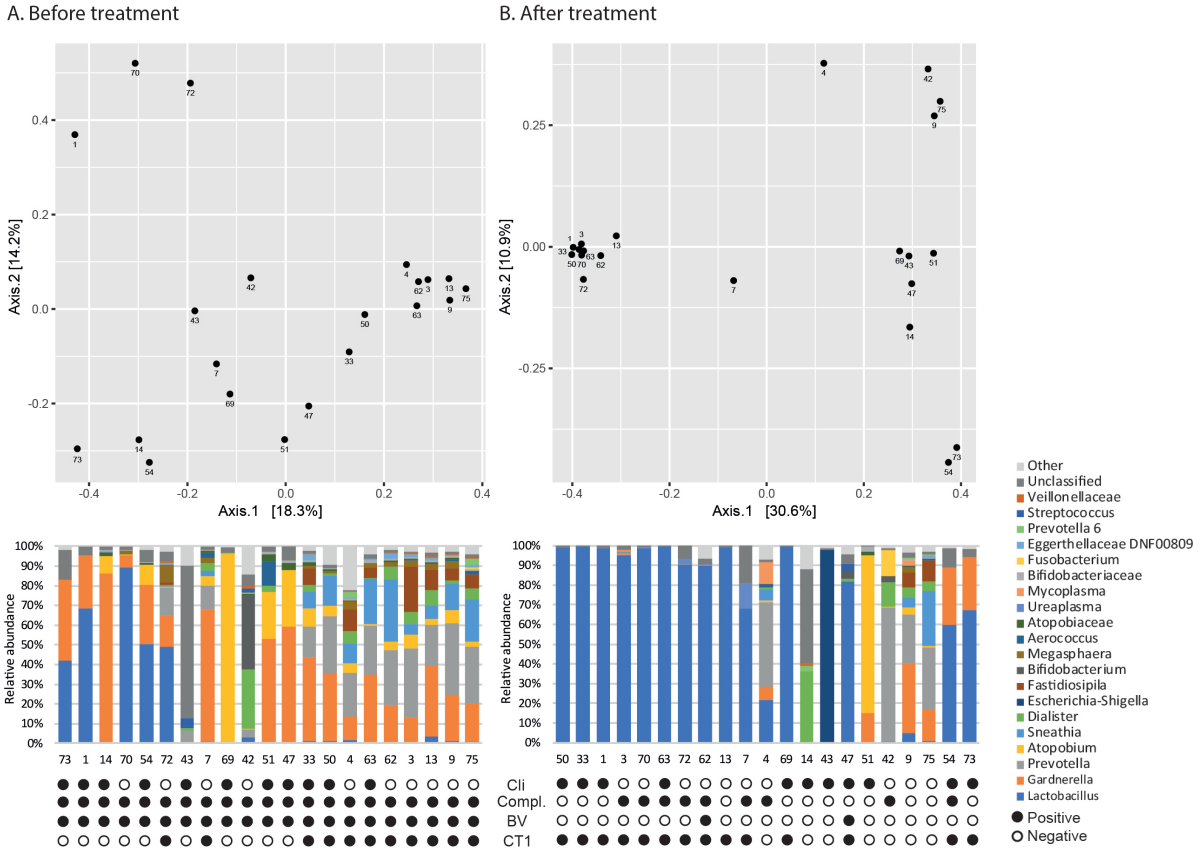

Figure 1. Principal coordinate analysis and taxonomic profiles of the vaginal microbiota before (**a**) and after (**b**) antibiotic treatment. Numbers indicate individual patients. Twenty taxa with highest average relative abundance are shown, abundances of all other taxa are summed and categorised as 'other'. For bargraphs, the subject order is matched to the subject order in the PCoA plots. Cli: clindamycin, Compl.: complaints of abnormal vaginal discharge, CT1: community type one, CT2: community type 2.

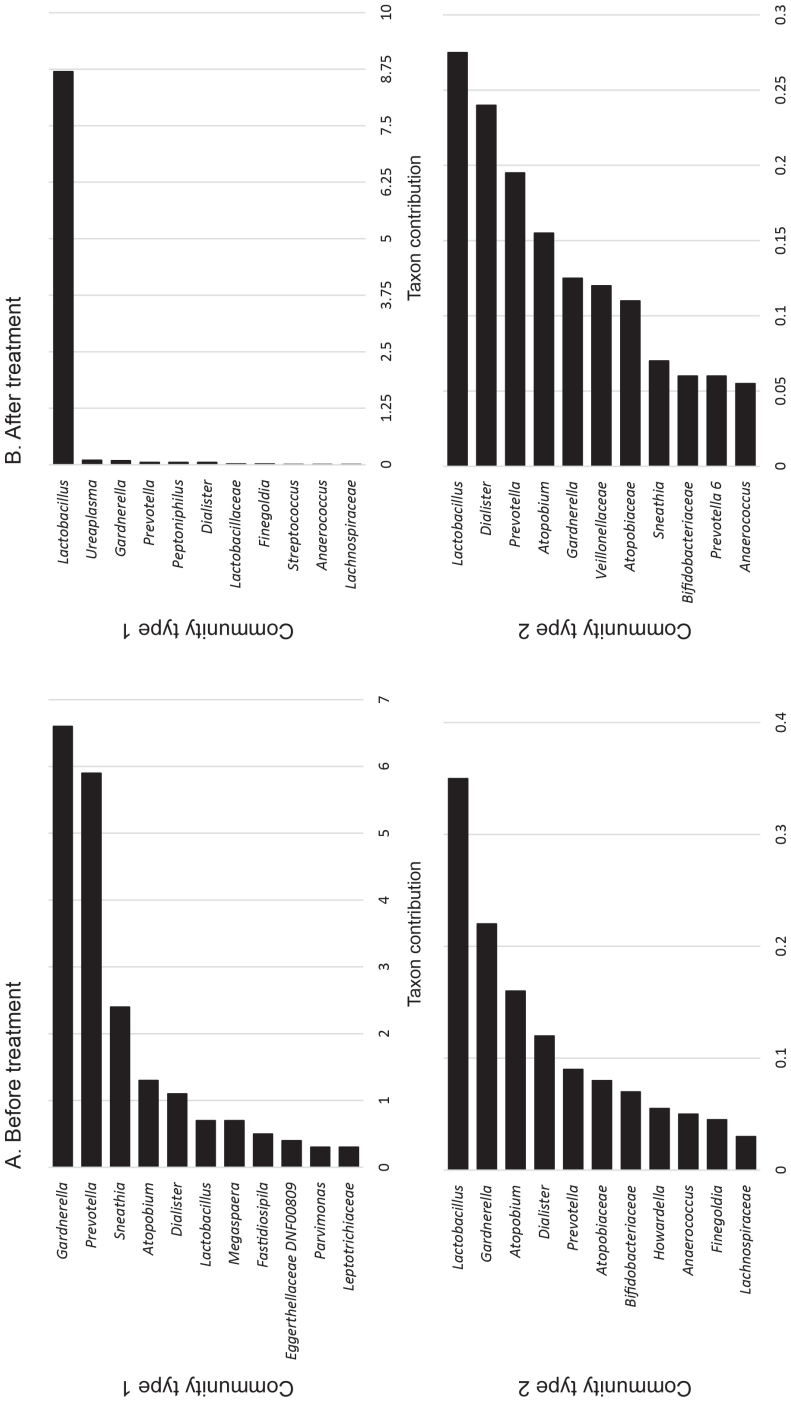

Figure 2. Vaginal microbiota community types before (**a**) and after (**b**) antibiotic treatment. For each community type, the 11 main driving bacterial taxa are shown.

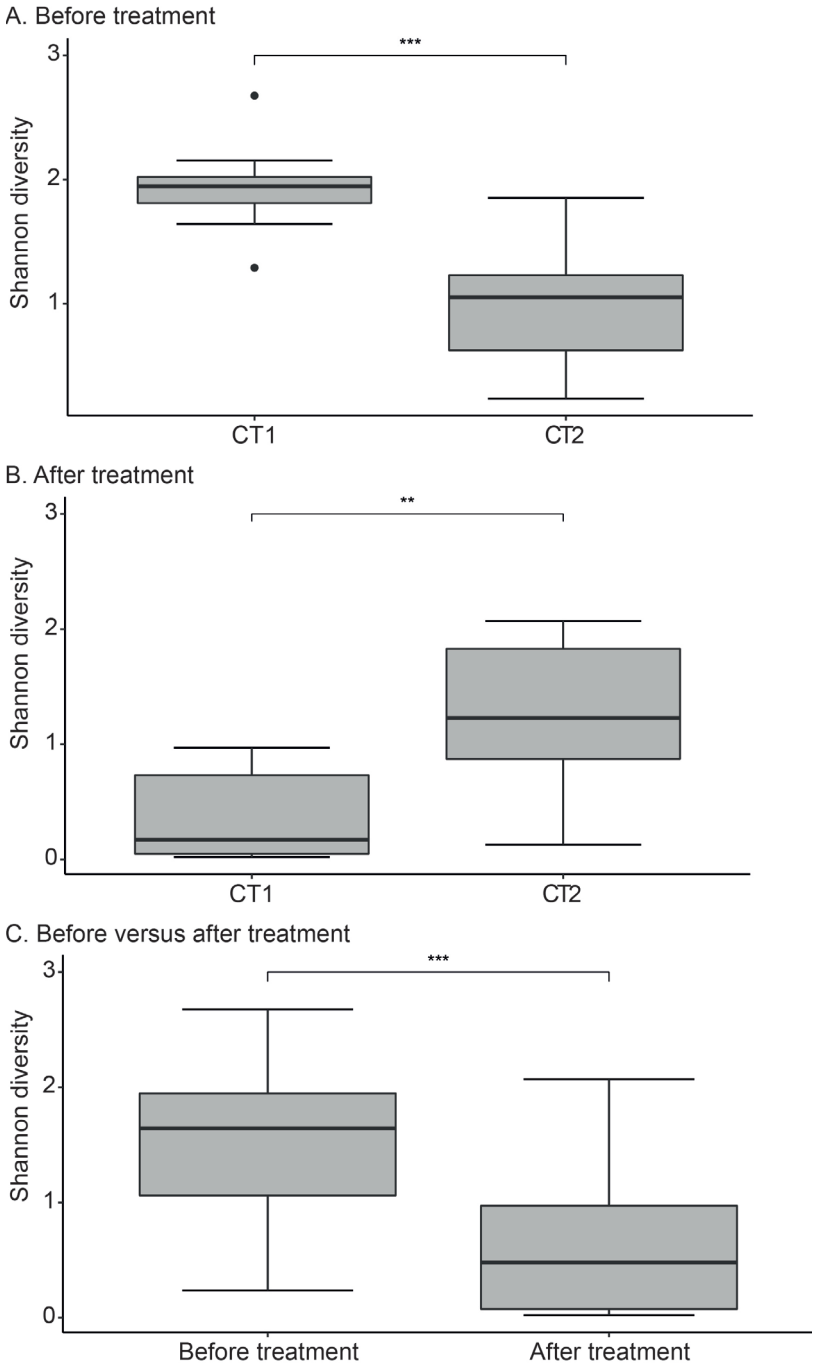

Figure 3. Bacterial diversity of each community type before treatment (**a**) and after treatment (**b**), and of all samples before and after treatment (**c**). Boxplots indicate the median, 25th and 75th percentile and whiskers indicate 1.5* interquartile range. *p < 0.05, **p < 0.01, ***p < 0.001. CT: community type.

Determining vaginal pH is more readily applicable, however, it most certainly simply reflects the abundance of lactic-acid producing bacteria, like *Lactobacillus*. Nowadays, PCR-based laboratory tests would be preferred for confirmation of the diagnosis (16). Except lactation and anticonception use, vaginal microbiota composition was not associated with patient demographics and lifestyle factors, which may be due to the relatively small subject size in combination with uniformity. It has previously been reported that host genetics, ethnicity, hormonal stage (e.g. menstruation cycle, menopause, pregnancy), sexual behaviour and hygiene practices, among others factors, influence vaginal microbiota composition (17-21).

After antibiotic treatment, nine women (43%) reported remaining complaints of abnormal vaginal discharge. Persisting complaints was more prevalent in women receiving metronidazole (70%) than in those receiving clindamycin (18%), which may be a result of differences in antibiotic spectrum and underlying conditions (e.g. pregnancy). To determine the potential influence of the microbiota on clinical outcome, vaginal microbiota composition before and/or after treatment were compared between patients with and without persistent complaints. The vaginal microbiota of women with persisting complaints contained a significantly higher relative abundance of *Ureaplasma* (Log2FoldChange = 8.73, pajd = 0.0008), but persisting complaints could not be associated with microbiota composition before treatment. *Ureaplasma* is a parasitic and saprophytic bacterium belonging to the Mollicutes class and is without cell wall, which results in intrinsic resistance to cell wall-targeting antibiotics like beta-lactam and glycopeptide antibiotics (22). *Ureaplasma* is intrinsically resistant to metronidazole, but usually susceptible to clindamycin (23). While carriage of *Ureaplasma* in urethra, cervix and vagina is common and generally asymptomatic, it has previously been associated with BV recurrence (24). Treatment outcome was not associated with the identified community types after treatment as persistent complaints were reported in 50% (7/14) and 29% (2/7) of women with vaginal microbiota composition belonging to the *Lactobacillus*-driven community type one or multiple species-driven community type two, respectively.

CONCLUSION

In conclusion, defining a (un)healthy vaginal microbiota state remains elusive, which challenges diagnosis and treatment of BV. Abnormal vaginal discharge and itching/irritation is most certainly not attributable to one or more specific bacteria, rather a disruption of the individual-specific mutualistic relationship of bacterial communities. Nevertheless, establishing universal markers for diagnosis and treatment of BV remains relevant. Herein, remaining complaints after treatment was more common in women who received metronidazole and was associated with increased relative abundance of the *Ureaplasma* genus, which may be considered when treatment fails.

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