

Treatment failure of bacterial vaginosis is not associated with higher loads of *Atopobium vaginae* and *Gardnerella vaginalis*

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Abstract

Purpose. Cervicovaginal *Atopobium vaginae* and *Gardnerella vaginalis* are strongly associated with bacterial vaginosis (BV) and are the main components of vaginal biofilms. The low efficacy of BV treatment with metronidazole may be due to the presence of such biofilms. Thus, the aim of this study was to compare the pretreatment cervicovaginal loads of *A. vaginae* and *G. vaginalis* for women who restored normal flora and those who persisted with BV after a full course of oral metronidazole.

Methodology. In this cross-sectional study, 309 reproductive-aged women were recruited in a primary health care service in Botucatu, Brazil. Cervicovaginal samples were tested for genital tract infections, microscopic classification of local microbiota and molecular quantification of *A. vaginae* and *G. vaginalis*.

Results. All the participants with concurrent cervicovaginal infections ($n=64$) were excluded. A total of 84 out of 245 (34.3 %) women had BV at enrolment and 43 (51.2 %) of them completed the treatment and returned for follow-up. Evaluation of the vaginal microbiota at follow-up showed that 29 (67.4 %) women restored normal vaginal flora, while 14 (32.6 %) still had BV. The pretreatment loads of *G. vaginalis* were lower in women with treatment failure ($P=0.001$) compared to those who successfully restored normal flora. The loads of *A. vaginae* did not differ between the groups.

Conclusion. Although *G. vaginalis* produces several virulence factors and its loads correlate positively with those of *A. vaginae*, higher cervicovaginal quantities of these bacteria are not associated with treatment failure of BV after oral metronidazole.

INTRODUCTION

Bacterial vaginosis (BV) is the most frequent type of abnormal vaginal flora in reproductive-aged women [1, 2]. Microbiologically, BV is defined by the shift from a normal lactobacilli-dominated flora to an excessive growth of anaerobic bacteria. In fact, several bacterial species have already been shown to be strongly associated with BV, such as *Atopobium vaginae*, *Gardnerella vaginalis*, *Prevotella* spp. and *Mobiluncus* spp., among others [3–5]. Women with BV have increased risk for acquisition for several sexually transmitted infections, such as human immunodeficiency virus (HIV), *Neisseria gonorrhoeae* and *Chlamydia trachomatis* [6–8].

The microbial features of BV are not completely understood, but studies have already shown that bacterial diversity [3–5]

and loads [9, 10] are increased in this condition compared to normal vaginal microbiota. When assessed individually, the loads of *A. vaginae* and *G. vaginalis* are also higher in BV [11]. Regarding *A. vaginae*, it is detected in nearly all women with BV and correlates positively with vaginal pH and Nugent scores [9]. In addition, independently of the presence of BV, higher vaginal loads of *A. vaginae* in pregnant women are associated with preterm birth [10]. Similarly, *G. vaginalis* can be detected in up to 100 % of women with BV [12, 13]. It has already been shown that strains of *G. vaginalis* isolated from women with BV are more virulent compared to strains recovered from women with normal microbiota [14]. Several virulence factors are produced by *G. vaginalis*, including hydrolytic enzymes such as sialidases and biofilms [15–18]. Sialidases produced by *G. vaginalis*

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Abbreviations: BV, bacterial vaginosis; CDC, Centers for Disease and Control and Prevention; CI, confidence interval.

degrade immunoglobulin A, impairing the local immune response [15]. Sialidases-producing strains of *G. vaginalis* also have increased capability of biofilm production [18, 19]. *A. vaginae* and *G. vaginalis* are the main components of vaginal biofilms [16, 17, 20].

Currently, one of the most common treatments for BV consists of a 1 g oral dose of metronidazole administered daily for 7 days. This regimen is in accordance with the guidelines from the Centers for Disease and Control and Prevention (CDC) [21]. This is the standard treatment for nearly all non-pregnant women attending gynaecology outpatient clinics in the Brazilian public health system. The overall prevalence of BV in this population is 30.0 % [2]. However, the metronidazole regimen has demonstrated low efficacy, with high rates of BV persistence and recurrence after treatment [22, 23]. The question of whether the poor response to metronidazole therapy is due to biofilm formation by *A. vaginae* and *G. vaginalis* and, consequently, their higher vaginal loads, is currently under discussion [24]. Other aspects of BV may also play a role in its treatment failure, such as the high diversity and heterogeneity of vaginal bacterial species [4, 5, 25]. Moreover, antimicrobial resistance genes have already been described in bacterial species associated with BV [26]. Further, other conditions of vaginal flora that are associated with loss of *Lactobacillus*, but are less responsive to metronidazole, such as aerobic vaginitis [27], may have been overlooked in some studies [28–30], leading to a false negative assessment of the treatment efficacy of metronidazole in such cases.

Taken together, these recent findings reinforce the importance of elucidating the microbiological features involved in the failure of BV treatment in order to seek strategies to increase its efficacy. Given that the growth of *A. vaginae* and *G. vaginalis* is expected to be higher in the presence of vaginal biofilms, the aim of this study was to compare their pretreatment cervicovaginal loads in women with BV who had successfully restored normal vaginal microbiota after oral metronidazole with those in women who had persisted with BV despite proper treatment.

METHODS

A total of 309 non-pregnant reproductive-aged women attending a public primary health care service in Botucatu, São Paulo, Brazil, were invited to participate in this prospective study. All of the women enrolled in the study attended outpatient clinics during a 6-month period in 2014 for routine cervical cancer screening (Pap test). Women were not included if they reported urinary loss, menstrual period, antibiotics in the previous 30 days and sexual intercourse in the last 48 h.

Sociodemographic, behavioural and clinical history data were assessed by applying an individual standardized questionnaire. During the physical examination for Pap test collection, the vaginal pH was measured by allowing 1 min of direct contact between pH strips (4.0–7.0; Merck, Darmstadt, Germany) and the vaginal wall. A potassium

hydroxide test was performed by placing a drop of 10 % KOH (v/v) on a swab with vaginal content. Results were then expressed as negative, doubtful and positive (whiff test). Microscopic analyses of vaginal wet-mount smears were performed to detect the presence of *Candida* sp.-like morphotypes, and *Trichomonas vaginalis*. The other vaginal smear was Gram-stained and used for vaginal flora classification according to the Nugent criteria as normal (score 0 to 3), intermediate (score 4 to 6) and BV (score 7 to 10) [31]. Endocervical samples were also taken to test for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using endpoint PCR and real-time PCR (RT-PCR), respectively, as described previously [27, 32, 33]. Finally, cervicovaginal samples were obtained by rinsing the vaginal wall using a 3 ml sterile NaCl 9.5 % solution according to a standardized technique [27].

The *A. vaginae* and *G. vaginalis* loads were determined by RT-PCR for cervicovaginal samples from women with BV and intermediate and normal flora that tested negative for other concurrent infections. Total bacterial DNA was extracted from the pellets of cervicovaginal samples using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions for Gram-negative and Gram-positive bacteria using a 100 µl final elution step. The reactions consisted of a 13 µl volume of Maxima SYBR Green/ROX mix (Fermentas, St Leon-Rot, Germany) and the pairs of primers specifically designed to amplify 16S rRNA regions of *A. vaginae* (81 bp) and *G. vaginalis* (332 bp) in separate reactions with 2 µl of DNA template [34, 35]. Amplification cycles were performed in LineGeneK (Bioer, China) equipment in 40 repetition cycles. The bacterial loads were calculated by the interpolation of cycle threshold values for each sample on the standard curve obtained with 10-fold serial dilution of plasmid DNA. The plasmids contained the amplified sequences of *A. vaginae* and *G. vaginalis* 16S rRNA genes that were constructed according to methods described elsewhere [11]. All samples were tested in duplicate and if a difference of more than one cycle threshold was observed between them, reactions were repeated. The mean values of the duplicates were calculated and the bacterial loads were expressed by number of copies ml⁻¹ of cervicovaginal sample. Final bacterial loads were obtained by multiplying the mean value by 50 in order to reach the elution volume of 100 µl that corresponded to 1 ml of the sample used for DNA extraction.

Treatment was offered to the 84 women who had BV at the time of enrolment. The treatment consisted of oral metronidazole 1 g administered daily for 7 days, as recommended by the CDC [21]. The 32 women with intermediate flora were only treated when they were symptomatic, and those who had no symptoms were advised to undergo re-evaluation within 30 days (this further visit was not part of this study protocol). Follow-up visits for BV-treated women were scheduled between 30 to 45 days after the end of treatment, depending on their menstrual period. During this follow-up visit, women were asked if they had completed the

treatment correctly, without skipping days. They were also asked if they had abstained from sexual intercourse during this period. Vaginal flora was then reassessed using Nugent's method [31]. Women who had taken an incomplete course of metronidazole or had had intercourse during treatment were not re-evaluated.

Comparison of discrete and continuous socio-demographics and clinical variables between the women with normal vaginal flora and BV was performed, respectively, by the Chi-squared and non-parametric Mann-Whitney tests. Comparison of the *A. vaginae* and *G. vaginalis* loads in patients classified as normal flora, intermediate flora and BV was performed using the non-parametric Kruskal-Wallis followed by Dunn's post-test. The Mann-Whitney non-parametric test was used to compare the bacterial loads of women who restored normal flora after metronidazole and those who persisted with BV. Spearman's correlation

coefficient was determined for the the *A. vaginae* and *G. vaginalis* loads of the study groups. All of these analyses were performed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA), with $P < 0.05$ considered to be significant.

RESULTS

From the total of 309 women initially recruited, those with vaginal candidosis ($n=26$, 8.4%), trichomoniasis ($n=1$, 0.3%), *C. trachomatis* ($n=22$, 7.1%), *N. gonorrhoeae* ($n=2$, 0.6%) or both *C. trachomatis* and *N. gonorrhoeae* infection ($n=1$, 0.3%) were excluded from the study (Fig. 1). Additionally, samples from 12 (3.9%) women were excluded because they were not suitable for analysis. Sociodemographic, behavioural and clinical data for the 245 women enrolled are shown in Table 1, according to the microscopic classification of the vaginal flora. Women

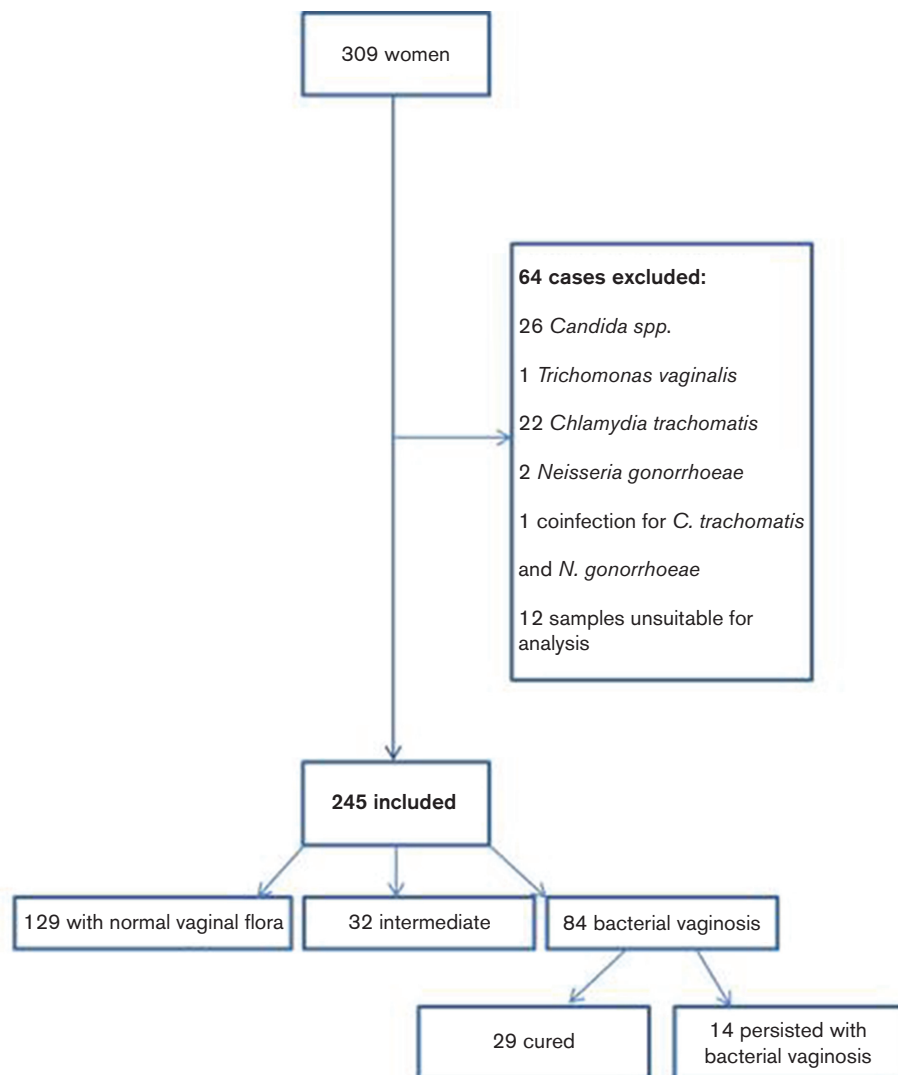


Fig. 1. Flow chart for participants in the study.

Table 1. Demographic, behavioural and clinical characteristics of the 245 women included in the study according to their vaginal flora type at enrolment

Continuous variables are expressed in median values (range) and categorized as total numbers (percentage). BV, bacterial vaginosis; STI, sexually transmitted infection.

Variables	Normal (n=129)	Intermediate (n=32)	Bacterial vaginosis (n=84)	P value
Age (years)*				
18–21 (n=34)	15 (44.1)	6 (17.6)	13 (38.2)	0.53
22–45 (n=193)	106 (54.9)	22 (11.4)	65 (33.7)	
46+ (n=18)	8 (44.5)	4 (22.2)	6 (33.3)	
Ethnicity*				
White (n=142)	82 (57.7)	19 (13.4)	41 (28.9)	0.10
Non-white (n=103)	47 (45.6)	13 (12.6)	43 (41.7)	
Marital status*				
Single (n=79)	36 (45.6)	6 (7.6)	37 (46.8)	0.01‡
Married/living with partner (n=166)	93 (56.0)	26 (15.7)	47 (28.3)	
Years at school‡	9 (0–16)	5.2 (1–11)	8 (0–15)	0.21
Paid job*				
Yes (n=133)	72 (54.1)	15 (11.3)	46 (34.6)	0.65
No (n=112)	57 (50.9)	17 (15.2)	38 (33.9)	
Smoking habit*				
Yes (n=47)	22 (46.8)	1 (2.1)	24 (51.1)	0.005§
No (n=198)	107 (54.0)	31 (15.7)	60 (30.3)	
Body mass index†	26.1 (16.4–49.7)	26.9 (15.6–38.1)	26.7 (16.0–53.0)	0.84
Number of sexual partners (1 year)*				
0 or 1 (n=216)	117 (54.2)	29 (13.4)	70 (32.4)	0.23
2 or more (n=29)	12 (41.4)	3 (10.3)	14 (48.3)	
Incidences of vaginal intercourse/week†	2 (0–7)	3 (0–7)	2 (0–7)	0.26
Previous BV*				
Yes (n=115)	53 (46.1)	17 (14.8)	45 (39.1)	0.15
No (n=130)	76 (58.5)	15 (11.5)	39 (33.9)	
Previous STI*				
Yes (n=21)	10 (47.6)	4 (19.0)	7 (33.3)	0.69
No (n=224)	119 (53.1)	28 (12.5)	77 (34.4)	
Consistent condom use*				
Yes (n=44)	18 (40.9)	7 (15.9)	19 (43.2)	0.23
No (n=201)	111 (55.2)	25 (12.4)	65 (32.3)	
Hormonal contraceptive (last 4 months)*				
Yes (n=170)	58 (58.0)	11 (11.0)	31 (31.0)	0.37
No (n=145)	71 (49.0)	21 (14.5)	53 (36.6)	
Vaginal pH*	4.4 (4.0–5.0)	4.7 (4.0–5.8)	4.7 (4.0–7.0)	<0.001§
Whiff test*				
Positive (n=75)	14 (18.7)	8 (10.7)	53 (70.7)	<0.001§
Doubtful or negative (n=170)	115 (67.6)	24 (14.1)	31 (18.2)	

*Chi-square test. $P < 0.05$ considered as significant.

†Kruskal–Wallis, followed by Dunn's post-test.

‡BV versus normal flora, BV versus intermediate flora.

§Differs among the three groups.

with normal flora ($n=129$, 52.6%), intermediate flora ($n=84$, 34.3%) and BV ($n=84$, 34.3%) did not differ for most of the variables evaluated, except with regard to the smoking habit, which was reported more frequently by women with BV. In addition, the subset of women who

were married or lived with a partner at enrolment were more likely to have normal flora. A positive whiff test and higher vaginal pH were more frequent in the BV group, followed by the intermediate and normal flora groups.

Cervicovaginal samples obtained at baseline had significantly higher loads of both *A. vaginae* and *G. vaginalis* for women with BV compared to those for women with intermediate and normal vaginal flora, as shown in Fig. 2. As shown in Table 2, there was a strong correlation between loads of *A. vaginae* and *G. vaginalis* in women with BV [Spearman $r=0.69$; 95 % confidence interval (CI)=0.55–0.79, $P<0.01$], while in women with intermediate flora this correlation was weak and non-significant (Spearman $r=0.25$; 95 % CI=0.11–0.56; $P=0.16$), and in women with normal vaginal flora it was weak but significant (Spearman $r=0.23$; 95 % CI=0.06–0.40; $P=0.01$).

Of the 84 women who had BV at enrolment, 46 (54.8 %) finished the treatment and returned for a follow-up visit. At follow-up, two participants reported that they had forgotten to take the medication at some point, and one had had sexual intercourse during treatment, and these subjects were

Table 2. Number of positive samples, [n (%)], Spearman correlation coefficient (r) and confidence interval (CI) [r (CI range)] for *A. vaginae* and *G. vaginalis* presented at enrolment by the 245 women included in the study

	Vaginal flora		
	Normal (n=129)	Intermediate (n=32)	Bacterial vaginosis (n=84)
<i>A. vaginae</i> positivity (%)	30 (23.3)	13 (40.6)	74 (88.1)
<i>G. vaginalis</i> positivity (%)	4 (31.0)	8 (25.0)	66 (78.6)
Spearman correlation for <i>A. vaginae</i> / <i>G. vaginalis</i>	0.23 (0.06–0.40)*	0.25 (0.11–0.56)	0.69 (0.55–0.79)*

* $P<0.01$.

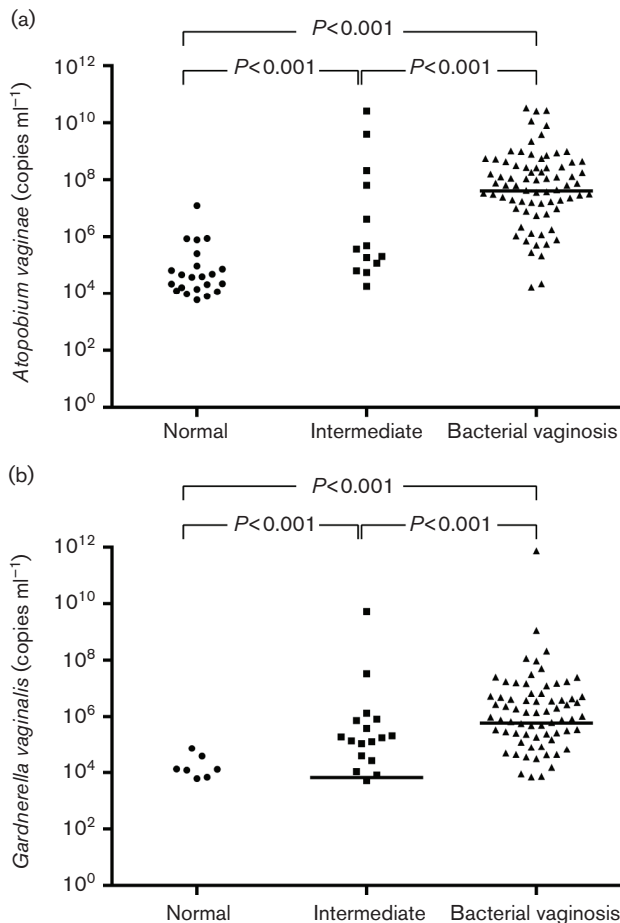


Fig. 2. Baseline cervicovaginal loads of *A. vaginae* and *G. vaginalis* in 129 women who presented normal vaginal flora, 32 with intermediate vaginal flora and 84 with bacterial vaginosis at enrolment. Horizontal bars represent the median values of copies ml^{-1} of cervicovaginal fluid. Comparison of bacterial load among the groups was performed using the Kruskal–Wallis test followed by Dunn's post-test

excluded from the analysis. The remaining 43 women who had completed the treatment properly were then divided in two groups: those who had shifted from BV to normal vaginal flora ($n=29$, 67.4 %) and those who had persisted with BV ($n=14$, 32.6 %). In the latter group, 13 women had Nugent scores higher than or equal to 7, and 1 had an intermediate vaginal flora (score=5). The participant with intermediate vaginal flora was included in the group of women who persisted with BV because she had a vaginal pH equal to 5.0 and continued to have abnormal vaginal discharge complaints. As shown in Fig. 3, the baseline cervicovaginal loads of *A. vaginae* did not differ between the group of women who restored normal flora after treatment and those who persisted with BV. However, the cervicovaginal *G. vaginalis* loads at baseline were significantly lower in women who persisted with BV after treatment. A strong correlation between *A. vaginae* and *G. vaginalis* cervicovaginal loads was observed in women with restored normal flora after metronidazole treatment (Spearman $r=0.68$; 95 % CI=0.42–0.84; $P<0.01$) and a slightly weaker correlation in women who persisted with BV (Spearman $r=0.58$; 95 % CI=0.05–0.85; $P<0.01$) (Table 3).

DISCUSSION

To the best of our knowledge, this is the first report to show that pretreatment cervicovaginal *G. vaginalis* loads are significantly lower in women who persist with BV after a proper regimen of oral metronidazole compared to those with restored normal flora after treatment. This finding is particularly relevant, since it may contribute to a better understanding of the role of the microbiological composition associated with BV persistence after metronidazole therapy. This regimen for BV treatment, with exclusive use of oral metronidazole, is still widely used in many developing countries, such as Brazil, in which BV prevalence is quite high [2].

When comparing the absolute loads of *A. vaginae* and *G. vaginalis* in different patterns of vaginal flora, the highest loads for both bacteria were observed in BV, followed by intermediate flora and normal vaginal flora. These results

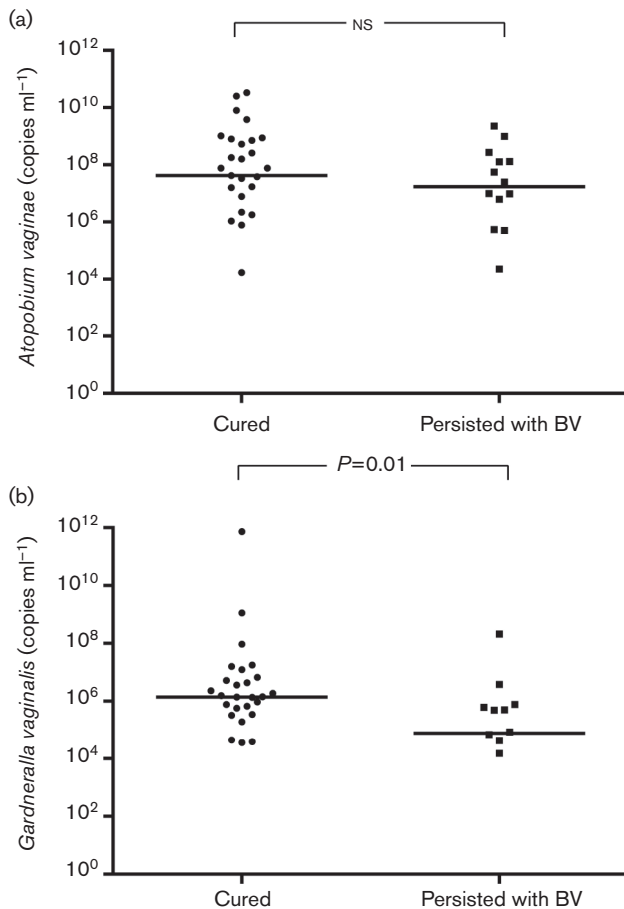


Fig. 3. Pretreatment cervicovaginal *A. vaginae* and *G. vaginalis* loads for 29 women with BV who restored *Lactobacillus*-dominant flora after metronidazole treatment and 14 BV-positive women who who persisted with BV after treatment. Horizontal bars represent the median values of copies ml^{-1} of cervicovaginal fluid. Comparison of bacterial load between the groups was performed using Mann-Whitney test.

were expected and they are in agreement with the findings of previous studies [36, 37]. Although *A. vaginae* and *G.*

vaginalis are well recognized as organisms associated with BV, they are also detected in the cervicovaginal samples of women with normal vaginal flora [38, 39], and this was also found in the current study. Thus, as has already been pointed out by other authors, the question of whether these species present more virulent genotypes when they are found in BV and, as a result, are more capable of disrupting vaginal flora, is a matter for discussion [14, 19, 40].

The current data showing the positive correlation found between *A. vaginae* and *G. vaginalis* in BV and normal vaginal flora are supported by previous findings on the co-detection of these two species in different types of flora [41]. However, in women with intermediate flora only a weak and non-significant correlation was found. This observation offers more evidence of the fact that the bacterial composition of intermediate flora is not classifiable as intermediate between normal and BV flora. In fact, the literature has consistently shown that the classification of intermediate flora is very heterogeneous, since other abnormal conditions of vaginal flora, such as aerobic vaginitis, may readily be classified as 'intermediate' when the Nugent criteria are used [13, 42]. As aerobic vaginitis scoring was not performed for these samples, its influence in intermediate and BV flora cannot be completely ruled out.

Even though *G. vaginalis* is one of the most frequently found species in BV [12, 40, 43], the current data show that women who have achieved a successful cure after treatment with metronidazole had significantly higher baseline loads of this organism compared to women who failed to restore lactobacilli-dominated flora (treatment failure). Thus, it is not possible to assign the treatment failure to an increased load of *G. vaginalis*. In fact, women with high loads of *G. vaginalis* respond better to metronidazole treatment. This finding highlights the importance of assessing the pathogenic diversity among the different strains of *G. vaginalis* for further determination of whether it plays a role in the treatment outcome.

On the other hand, the bacterial diversity of vaginal flora may also be a factor that influences BV treatment. What has been learnt so far is that the correlation between *G. vaginalis* and *A. vaginae* is weaker in the group of women who persisted with BV after treatment. Thus, it can be hypothesized that other micro-organisms may be interfering with this synergism and establishing a bacterial community that is less susceptible to metronidazole, leading to treatment failure. In a previous study performed by Bradshaw *et al.* [12], the co-detection of *G. vaginalis* and *A. vaginae* was associated with recurrent BV. However, despite showing quantitative data for *A. vaginae* and *G. vaginalis*, the former study did not provide the baseline pretreatment loads for these bacteria. The fact that the lower sensitivity of *A. vaginae* to metronidazole favours a cure in women with higher *G. vaginalis* loads could also be discussed, as a higher proportion of *A. vaginae* compared to *G. vaginalis* may be triggering treatment failure in such cases [44].

Table 3. Number of positive samples, [n (%)], Spearman correlation coefficient (*r*) and confidence interval (CI), [*r* (CI range)] for *A. vaginae* and *G. vaginalis* presented pretreatment by the 45 women with BV who completed the metronidazole course and returned for follow-up.

	Treatment outcome	
	Normal flora (n=29)	Persisting bacterial vaginosis (n=14)
<i>A. vaginae</i> positivity (%)	24 (85.7)	13 (92.9)
<i>G. vaginalis</i> positivity (%)	23 (82.1)	10 (71.4)
Correlation of <i>A. vaginae</i> / <i>G. vaginalis</i> loads	0.68 (0.42–0.84)*	0.58 (0.05–0.85)*

* $P < 0.01$.

The development of more refined molecular methods for determining vaginal flora allowed the recognition of new bacterial species associated with BV, such as BV-associated bacteria (BVAB)-1, BVAB-2 and BVAB-3, which belong to the *Clostridiales* order. It was recently suggested that these micro-organisms can produce endospores that could lead to fast recolonization after the antibiotics regimen ends [45]. In addition, these newly recognized species are associated with *Mobiluncus* sp. suggestive morphotypes in BV. *Mobiluncus* sp. strains that have already showed significant resistance rates to metronidazole *in vitro* [46, 47].

A previous study by this group that used flow cytometry showed that the total bacterial count in BV does not determine the outcome to metronidazole treatment [48]. Thus, given that the bacterial count is not associated with BV treatment failure, while the *G. vaginalis* loads decrease in such cases, the poor treatment outcome may be associated with the replacement of *G. vaginalis* by other bacteria in the niche it had occupied in the BV bacterial community. Further, as the higher loads of *G. vaginalis* and *A. vaginae* were shown to be useless in predicting treatment failure for BV, future studies are essential to determine the microbiological aspects involved in this troublesome issue. Then, more efficient alternatives may be developed to protect these women from the serious consequences of persisting with abnormal vaginal flora for long periods of time.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This study was reviewed and approved by the Ethics Committee Board of Botucatu Medical School, São Paulo State University (no. 478.483), and all participants provided a signed consent form.

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