

Original Article

Assessment of vaginal microbiota in Brazilian women with and without bacterial vaginosis and comparison with Nugent score

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Abstract

Introduction: Bacterial vaginosis (BV) is characterized by the depletion of *Lactobacillus* spp. population and increase of other species, especially *Gardnerella vaginalis* and *Atopobium vaginae*. This study aimed to investigate the vaginal microbiota structure of Brazilian women with and without BV according to Nugent Score and to assess the correlation among Nugent score and the quantification of BV-associated bacteria.

Methodology: Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) assay was employed to characterize the vaginal microbiota structure. Quantification of *Lactobacillus* spp., *G. vaginalis*, *A. vaginae*, *Mobiluncus* sp. and *M. hominis* were determined by quantitative real-time PCR (qPCR).

Results: Clustering by PCR-DGGE revealed differences in microbial structure of the different patient groups. *Gardnerella vaginalis*, *A. vaginae*, *M. hominis* and *Mobiluncus* sp. were detected at high loads in BV-associated microbiota. Quantification of *Lactobacillus* spp. showed an inverse correlation with Nugent score while the loads of *G. vaginalis*, *A. vaginae*, *M. hominis* and *Mobiluncus* sp. indicated a direct correlation with this method.

Conclusions: Despite Nugent score is considered the gold standard for BV diagnosis, qPCR stands out as a useful tool for bacteria quantification and an alternative for BV diagnosis. Vaginal microbiota is a complex microbial community although there is a common core among BV and non-BV women. Investigation of vaginal microbiota structure may contribute to the development of tools for diagnosis improvement and therapeutic regimen optimization.

Key words: bacterial vaginosis; *Gardnerella vaginalis*; *Lactobacillus*; vaginal microbiota; Nugent score.

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Introduction

Microorganisms inhabiting the vaginal environment are constantly submitted to selective pressures, like immune response and pH, which contribute to ecosystem balance and modulate its distribution on resident microbiota [1]. Therefore, the health status of female genital tract is dependent on the vaginal bacterial community [2]. In healthy individuals, the vaginal microbiota comprises mainly *Lactobacillus* species, particularly *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* [3-5]. Lactobacilli express different characteristics, such as specific adhesion to surface epithelial cells and antimicrobial substances, which make difficult the establishment of pathogenic microorganisms. They also compete with potentially pathogenic bacteria for space and nutrients [4,6,7]. Moreover, healthy vaginal microbiota may also be composed by pathogenic bacteria, such as *Gardnerella*

vaginalis, in low numbers, coexisting in equilibrium with *Lactobacillus* species [8].

Bacterial vaginosis (BV) is a multifactorial syndrome characterized by depletion of *Lactobacillus* spp. population and proliferation of anaerobic and facultative microorganisms. It can be observed vaginal pH increase and malodorous vaginal discharge, among other symptoms [9,10] and the polymicrobial etiology is mainly marked by the presence of *G. vaginalis*, *Mobiluncus* sp., *Mycoplasma hominis* and *Atopobium vaginae* [9,11]. However, it is still unclear whether the primary event for BV onset is the loss of *Lactobacillus* species or the acquisition of complex microbial communities found in this syndrome, or both [5,9]. In this context, vaginal microbial structure characterization in BV and non-BV women is important to understand the complex etiology of this disease,

especially considering the non-*Gardnerella vaginalis* species [1,12,13].

Culture-dependent methods do not allow identification of uncultivable bacteria and make difficult the determination of bacterial load associated with clinical disease in complex bacterial communities [1,14]. In contrast, culture-independent molecular methods have been proven to be efficient tools for phylogenetic diversity studies and understanding the dynamics of these communities [15-18]. Molecular assays based on genetic fingerprinting and 16S rDNA sequence analysis have enabled bacterial diversity investigation in different microbial communities [17,19]. Recent advances have been achieved by culture-independent techniques, allowing association of bacteria species with BV for the first time, such as *A. vaginae* [17,20-22]. Polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) have been used to investigate BV-associated bacteria composition, while real-time PCR (qPCR) stands out as a useful tool for bacteria quantification [23-25]. In addition, qPCR schemes have recently been developed as an alternative for BV diagnosis [23,24].

Currently, clinical diagnosis of BV is established according two protocols, Amsel's criteria and Nugent score, which include clinical and laboratory observations, respectively [5]. Nugent score is based on standardized Gram-stain interpretation in which vaginal smears are graded on a 10-point scale according the presence of different bacterial morphotypes [26]. This method is considered a reliable diagnostic approach from the viewpoint of reproducibility [27] and nowadays, it is considered the gold standard for BV diagnosis [10]. However, this system is a qualitative method, requires specialized training and does not provide information of some BV-associated bacteria, like *A. vaginae* and *M. hominis* [5,23].

This study aimed to investigate the vaginal microbiota structure of Brazilian women, classified with and without BV according to Nugent Score, regarding Bacteria domain and Firmicutes and Actinobacteria phyla, to determine the load of *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus* sp., *Lactobacillus* spp. and *Mycoplasma hominis* and to assess the relationship among Nugent score and bacterial quantification.

Methodology

Population and study design

This prospective cross-sectional study comprised 81 women at reproductive age, randomly selected from public and private health services of Juiz de Fora, MG,

Brazil, between April 2011 and April 2012, and was approved by the Federal University of Juiz de Fora Ethics Committee. The following inclusion criteria were considered: symptomatic or asymptomatic patients undergoing routine Pap test, who did not use systemic antibacterial or antifungal drugs in the past 30 days, did not have sexual relationship within five days prior the examination, did not use topical vaginal cosmetics or sanitizers, and agreed to participate by signing the written informed consent. Exclusion criteria were pregnancy and patients with clinical and laboratory diagnosis of fungal or protozoan cervical-vaginal infections. Vaginal specimens were collected during clinical examination. Sterile swabs were saturated with vaginal discharge and placed in a test tube containing 1.0 mL of *Gardnerella* Transport Medium (protease peptone #3, 1.35%; glycerin, 10%, pH 6.8 – Vetec, Rio de Janeiro, Brazil). Swabs were placed into ice box and immediately (up to 60 minutes) sent to the Laboratory of Bacterial Physiology and Molecular Genetics for further experimental procedures. Smears were prepared on glass slides, Gram-stained and examined under oil immersion objective (1000X magnification) for specific bacterial morphologies observation, according to Nugent score guidelines, according to the Table 1 [26].

DNA extraction

Genomic DNA from vaginal specimens was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following manufacturer's recommendations with minor modifications. After addition of 20 µL proteinase K (Qiagen, Hilden, Germany) and 200µL of AL buffer (Qiagen, Hilden, Germany), samples were incubated at 56°C for 30 minutes. Genomic DNA samples were stored at -20°C for additional analysis. Total DNA was standardized at 90 ng/µL and 10 ng/µL for DGGE and qPCR assays, respectively.

Amplification and Denaturing Gradient Gel Electrophoresis (DGGE)

Nested-polymerase chain reaction (nested-PCR) followed by DGGE was performed to evaluate the vaginal microbiota structure. In the first round of PCR, the primers F984GC (5'-gc-AACGCGAAGAACCTTAC 3')/R1492 (5'-TACGG(C/T)TA CCTTGTTACGACTT 3'), F243 (5'-GGATGAGCCCCGCGCCTA 3')/R1492 and BLS342F (5'-CAGCAGTAGGGAATCTTC 3')/R1392 (5'-ACGGGCGGTGTGTACA 3') were used to amplify 16S rDNA gene regions specific to Bacteria

domain, Actinobacteria and Firmicutes phyla, respectively [28,29]. Primer F984GC contained a GC-clamp at the 5' end [28]. Following first PCR, amplicons were used as DNA template in the second reaction using the primers F984GC/R1378 (5' CGGTGTGTACAAGGCCCGGAACG 3') [28]. Each PCR consisted of 25 µL reactions with DNA template, 10 µM of each forward or reverse primer, and 1X PCR Master Mix (Promega, Madison, USA). Positive control was composed by a mixture of bacterial DNA extracted from reference strains (*G. vaginalis* ATCC 49145, *Staphylococcus aureus* ATCC 35591, *Bacteroides fragilis* ATCC 43859, *Enterococcus faecalis* ATCC 51299, *Enterococcus faecium* ATCC 35667), from bacteria previously characterized by DNA sequencing (*Lactobacillus johnsonii*), or purified and characterized from specific 16S rDNA PCR reactions (*Mobiluncus* sp. and *Atopobium vaginae*). Before DGGE assays, PCR products were analyzed by electrophoresis in a 1.2% agarose (Sigma-Aldrich, St. Louis, USA) and gel stained with ethidium bromide (Promega, Madison, USA). Marker 100bp DNA ladder (Promega, Madison, USA) was used as molecular weight standard. DGGE was carried out using the DCode. Universal Mutation Detection System apparatus (BioRad, Hercules, USA). Amplicons from second round of nested-PCR were loaded onto an 8% polyacrylamide (Sigma-Aldrich, St. Louis, USA) gel with denaturing gradient gel set as 45-65% urea/formamide (Promega, Madison, USA). Electrophoresis was performed in 1 X TAE buffer, at 50 V, for 16 hours, at a constant temperature of 60°C. DNA fragments were stained with 1 X SYBR Gold (Invitrogen, Eugene, USA). Gel image was visualized and documented on a UV transilluminator (ImageQuant 100, GE Healthcare, Piscataway, USA) assembled with

a digital camera (Olympus LENS ED SP-500 UZ, Tokyo, Japan).

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) assays for *G. vaginalis*, *A. vaginae*, *Mobiluncus* sp., *Lactobacillus* spp. and *M. hominis* were SYBR green-based and performed using the Rotor-Gene Q detection system (Qiagen, Hilden, Germany). Reaction mixture (25 µL) contained 1X Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) and DNA template. Primer sequences targeting *G. vaginalis* *cpn60* gene (F- 5' CGCATCTGCT AAGGATGTTG 3'/R- 5'CGCATCTG CTAAGGATGTTG 3') and 16S rDNA of *A. vaginae* (F- 5'F-CCCTATCCGCTCC TGATACC 3'/R- 5' CCAAATATCTGCGCATTCA 3'), *Lactobacillus* spp. (F- 5'ATGGAAGAACACCAGTGGCG 3'/R- 5' CAGCACTGAGAGGCGGAAAC 3'), *Mobiluncus* sp. (F- 5'CCACGCTGTAACGTTGGGAA 3'/R- 5' TGGCCCATC TCTGGAACCA 3') and *M. hominis* (F- 5' AGGTTAGCAATAACCTAGCGGCGA 3'/R- 5' TTACAGCGCCTTTCACAACG 3') were used in the qPCR assays [30-33]. Samples were assayed in duplicate and average values were used to calculate bacterial quantification. Negative controls were included. Standard curves of *G. vaginalis* and *M. hominis* were generated using genomic DNA of *G. vaginalis* ATCC 14019 and *M. hominis* ATCC 15488. Standard curve for *Lactobacillus* quantification was constructed using *L. johnsonii* genomic DNA. Standard curves for *A. vaginae* and *Mobiluncus* sp. quantification were generated from a pGEM-T plasmid construct, cloned with a fragment of 16S rDNA gene of each bacterium. Competent *Escherichia coli* JM109 (Promega, Madison, USA) was used for cloning. All

Table 1. Nugent scoring system for Gram-stained vaginal smears.

Morphotypes	Number of microorganisms/immersion field	Score*
<i>Lactobacillus</i> spp.	> 30	0
	5 – 30	1
	1 – 4	2
	1	3
	0	4
	0	0
Curved Gram variable rods	1 – 4	1
	5	2
	> 30	4
	5 – 30	3
<i>Gardnerella vaginalis</i> / <i>Bacteroides</i> spp.	1 – 4	2
	1	1
	0	0

* Morphotypes are scored as the average number see per oil immersion field. Interpretation is based in the sum of average of each morphotype score: 0-3: normal microbiota; 4-6: intermediate microbiota; 7-10: bacterial vaginosis.

standard curves were constructed using serial 10-fold dilutions ($10^1 - 10^7$ copies/ μL) to determine bacterial concentration in copies/ μL . Results were expressed as DNA copies/ μL and then converted to copies/mL considering the initial volume of vaginal specimens used for metagenomic DNA extraction. Genomic DNA of *G. vaginalis* ATCC 14019, *M. hominis* ATCC 15488 and sequenced vaginal isolated *L. johnsonii* were extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA). Plasmid DNA containing the 16S rDNA gene of *A. vaginae* and *Mobiluncus* sp. were extracted using the AxyPrep Plasmid Miniprep Kit (Axygen Biosciences, Union City, USA).

Data analysis

Comparisons of DGGE gels profiles were carried out using the BioNumerics software version 7.1 (Applied Maths, Ghent, Belgium). Resulting patterns were compared using Dice similarity coefficient and the matrix generated was clustered by the Unweighted Pair Group Method with Arithmetic (UPGMA). Richness and Shannon-Weaver (H) diversity index were calculated using the PAST 3 software (Natural History Museum, University of Oslo, Oslo, Norway). Richness was estimated based on a binary matrix, in which the

presence of a band, which corresponds to each operational taxonomic unit (OTU), is coded as 1 and the absence as 0. Student t test was carried out using the software GraphPad Prism 5.0 (GraphPad Software, San Diego, USA) in order to analyze richness and diversity data among BV and non-BV patient groups ($p < 0.05$). Bacterial quantification results were analyzed by Student t test and significance level was set as $P < 0.05$. Relationship among Nugent score and bacterial quantification was evaluated through linear regression and Pearson correlation. *R*-value close to 1 was considered a strong positive correlation. All comparisons were performed using the GraphPad Prism 5.0 software. Intermediate group was not considered in the statistical analysis due to the small number of observations ($n = 3$).

Results

Classification of BV and non-BV women

A total of 81 patients were enrolled in this study and categorized according to Nugent score guidelines in three patient groups, BV, non-BV and intermediate groups [26]. BV patient group was composed by 36 women, non-BV patient group was represented by 42 women and 3 subjects were classified as intermediate group.

Figure 1. Clustering analysis of PCR amplified bacterial 16S rDNA DGGE fingerprints using metagenomic DNA extracts from vaginal specimens. (A) Bacteria domain (41 non-BV and 35 BV individuals) (B) Phylum Actinobacteria (39 non-BV and 34 BV individuals) and (C) Phylum Firmicutes (34 non-BV and 34 BV women).

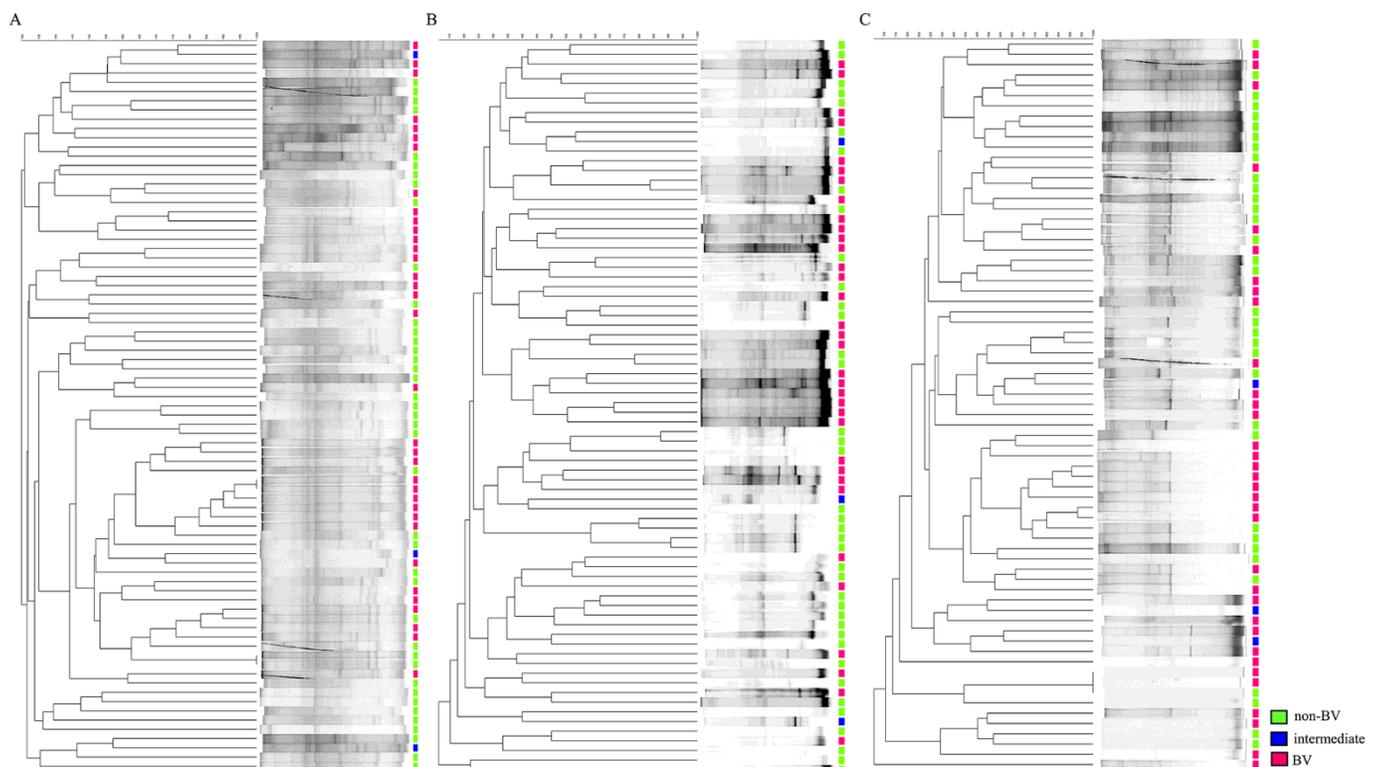


Figure 2. Richness (A) and diversity (B) analysis of Bacteria domain and Actinobacteria and Firmicutes phyla of vaginal specimens. Bacteria domain: 41 non-BV and 35 BV individuals; Actinobacteria: 39 non-BV and 34 BV individuals; Firmicutes: 34 non-BV and 34 BV individuals. Each bar represents mean + SEM; *p* values < 0.05 were considered significant.

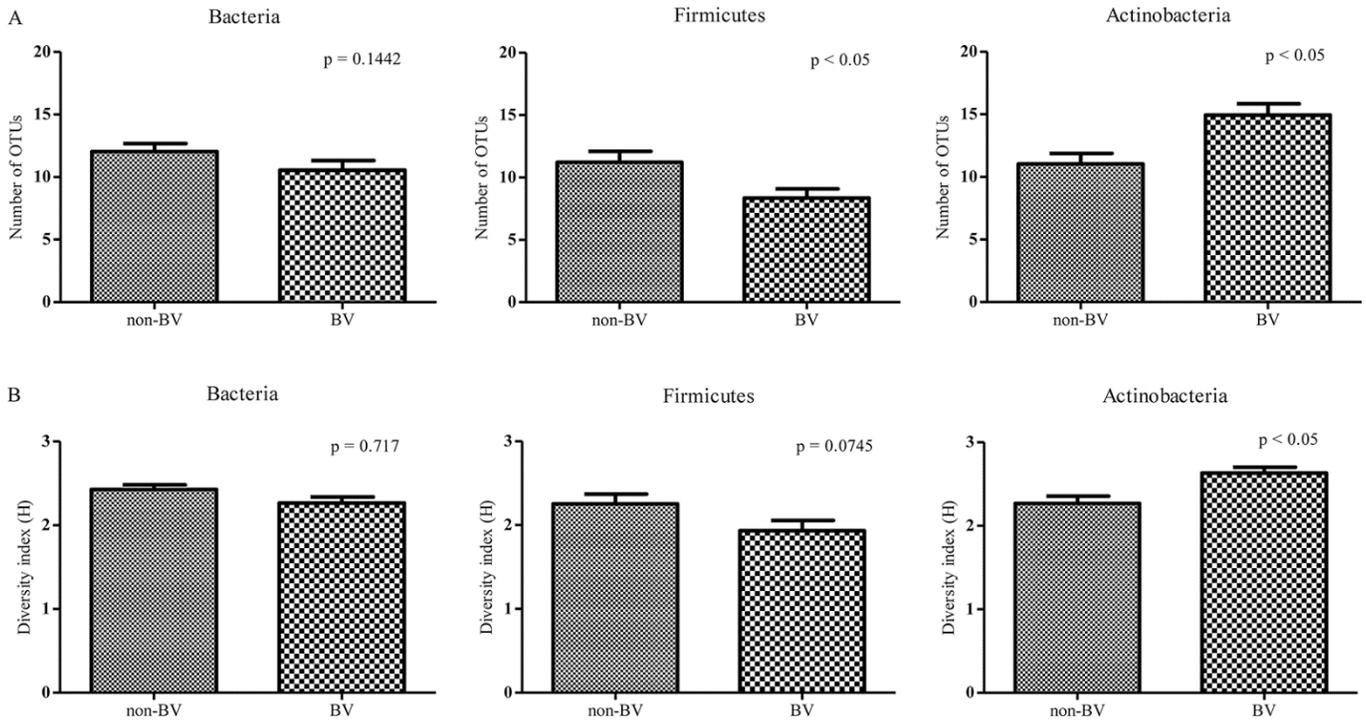
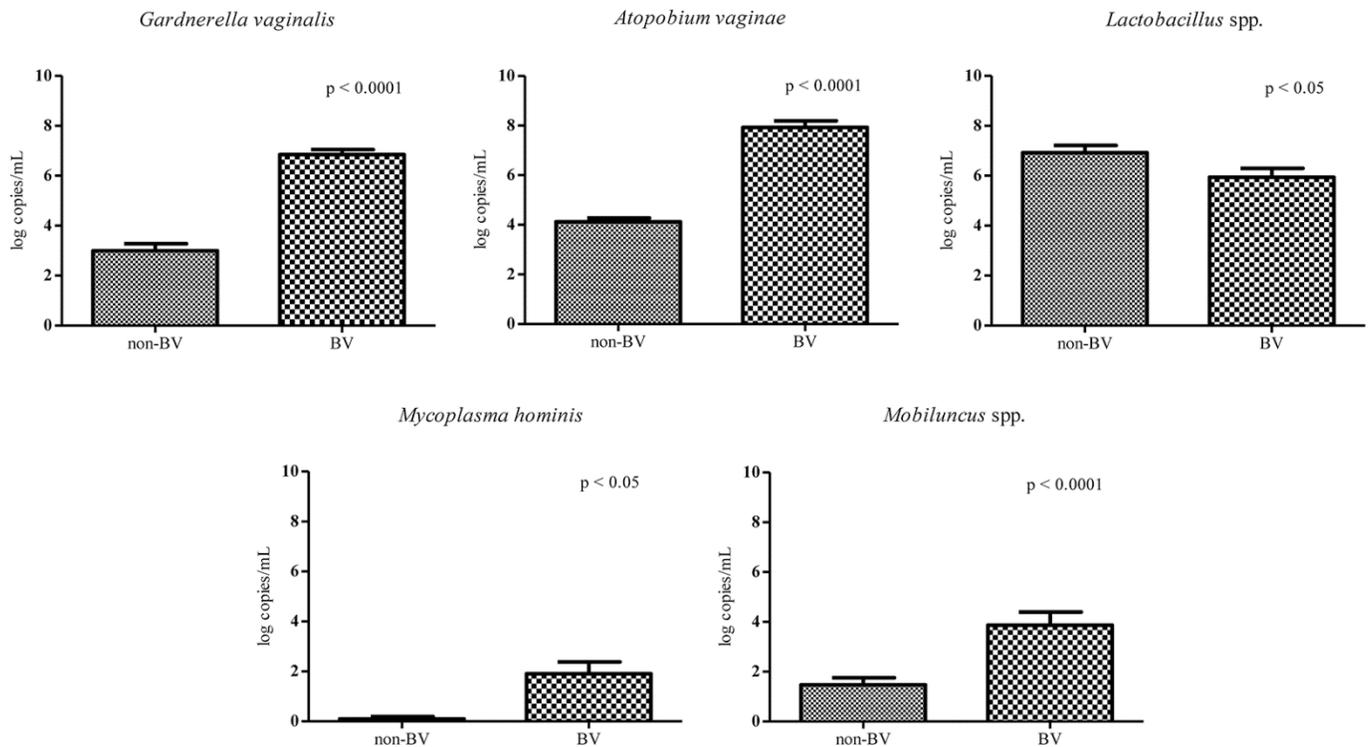


Figure 3. Quantification of *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus* spp., *Lactobacillus* spp. and *Mycoplasma hominis* recovered from the vaginal specimens included in this study. Quantification of *G. vaginalis*, *A. vaginae*, *Mobiluncus* spp. and *Lactobacillus* spp. was performed on 42 non-BV and 36 BV specimens; *M. hominis* quantification was carried out on 35 non-BV and 33 BV specimens. Each bar represents mean + SEM; *p* values < 0.05 were considered significant.



Assessment of BV and non-BV vaginal microbiota structure

DGGE fingerprinting from the analysis of Bacteria domain, Actinobacteria and Firmicutes phyla revealed differences in the microbial structure between women groups. Actinobacteria and Firmicutes phyla were chosen because species included in these phyla have strong association with BV. Cluster analysis showed that profiles of patients having the same health status (i.e. BV and non-BV) tended to be together, however in separate clusters (Figure 1A, 1B and 1C). The number of bands in the gel (i.e. filotypes or operational taxonomic units - OTUs), provides an approximate indication of species richness. Richness data taken together with *Shannon-Weaver* diversity index (H) results showed BV and non-BV vaginal microbiota have complex profiles. Among BV and non-BV women, there was no significant difference for richness and diversity in relation to Bacteria domain. Regarding Firmicutes phylum, BV and non-BV groups were statistically different ($p < 0.05$) with respect to richness. However, there was no significant difference for diversity. Actinobacteria was the phylum which BV and

non-BV groups were statistically different ($p < 0.05$) for both richness and diversity (Figure 2A and 2B).

Quantification of the main BV-associated bacteria and comparison with Nugent Score

qPCR evaluation showed significant differences in the quantification of *G. vaginalis*, *A. vaginae*, *Mobiluncus* sp., *M. hominis* and *Lactobacillus* spp. among BV and non-BV women ($p < 0.05$) (Figure 3). *Gardnerella vaginalis* was detected in all subjects (36 of 36) of BV patient group at high loads ($\geq 10^4$ copies/mL) and in 78.6% (33 of 42) of non-BV women at minor concentrations. *Atopobium vaginae* was detected in all BV individuals (36 of 36) at high concentrations ($\geq 10^5$ copies/mL) and in 100% (41 of 41) of non-BV women at minor loads. Among some BV women, in which *A. vaginae* concentration was low, a raised load of *G. vaginalis* was detected, agreeing with BV status defined by Nugent Score. *Mobiluncus* sp. was detected in 94.4% (34 of 36) and 85.7% (36 of 42) of BV and non-BV women, respectively, although at minor concentrations ($\leq 10^4$ copies/mL) in the last one *Lactobacillus* spp. was identified in 97.2% (35 of 36) of BV subjects and in 97.6% (41 of 42) of non-BV women,

Figure 4. Correlation analysis between Nugent score and quantification of *Gardnerella vaginalis*, *Atopobium vaginae*, *Mycoplasma hominis*, *Mobiluncus* spp. and *Lactobacillus* spp. in the vaginal specimens (BV, intermediate and non-BV). For *G. vaginalis*, *A. vaginae*, *Mobiluncus* spp. and *Lactobacillus* spp., 36 BV, 3 intermediate and 42 non-BV specimens were evaluated; for *M. hominis*, 35 non-BV, 2 intermediate and 33 BV specimens were assessed. p values < 0.05 were considered significant; R values of ≥ 0.5 were considered a positive correlation.

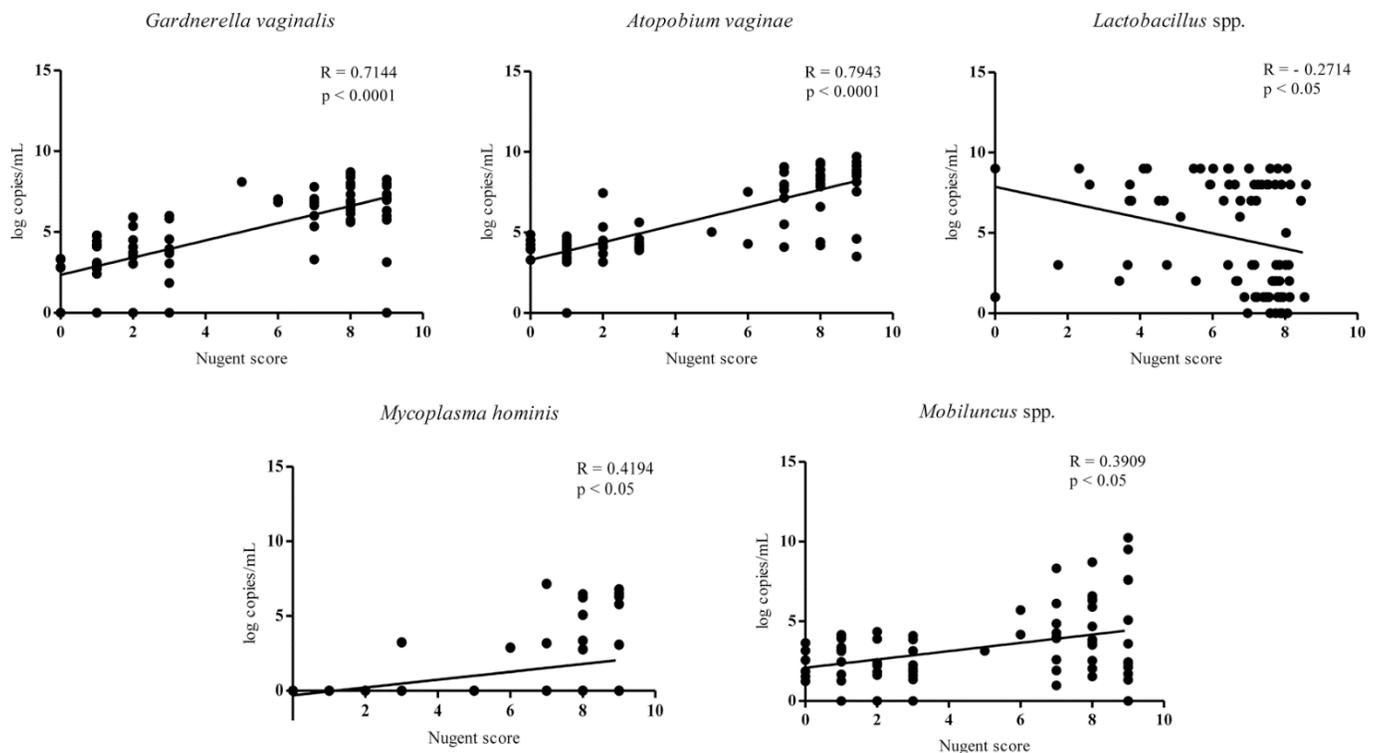
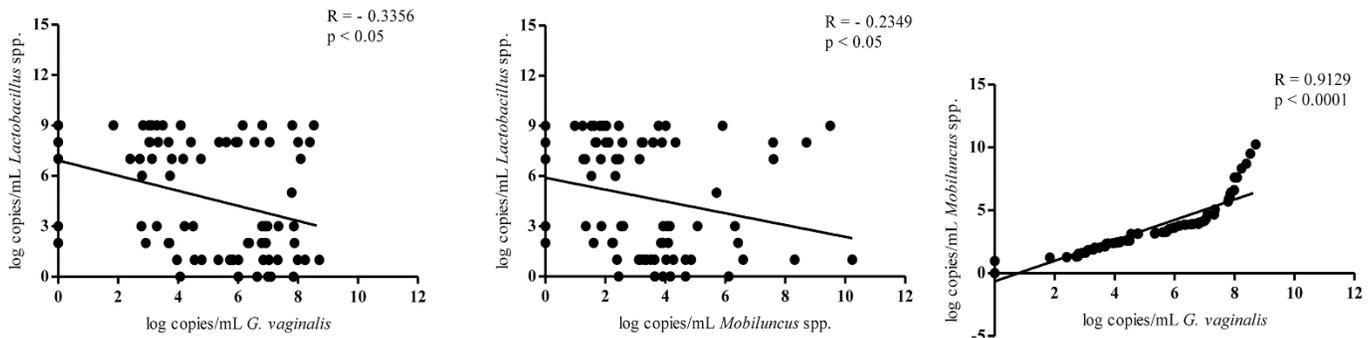


Figure 5. Correlation analyses between *Gardnerella vaginalis*, *Mobiluncus* spp. and *Lactobacillus* spp. quantifications considering the 81 vaginal specimens (BV, intermediate and non-BV) evaluated in this study. p values < 0.05 were considered significant; R values of ≥ 0.5 were considered a positive correlation.



with similar concentration ranges in both groups (0-10⁸ copies/mL for BV group and 10¹-10⁸ copies/mL for non-BV group). However, 85.7% (36 of 42) of non-BV women presented quantification results of $\geq 10^6$ copies/mL while 61.1% (22 of 36) of BV subjects presented bacterial loads above this cutoff. *Mycoplasma hominis* was detected in 2.4% (1 of 42) of non-BV woman (10³ copies/mL) and in 36.1% (13 of 36) of BV patient group (10³-10⁷ copies/mL). *Lactobacillus* spp., *G. vaginalis* and *A. vaginae* were concomitantly detected at high concentration ($\geq 10^5$ copies/mL) in 9.5% (4 of 42) of non-BV and in 63.9% (23 of 36) of BV women. Bacterial quantification results of intermediate patients were more similar to those of BV women once high loads of *G. vaginalis*, *A. vaginae* and *Mobiluncus* sp. were detected.

Regarding the comparison among bacterial quantification and Nugent score, *Lactobacillus* spp. was negative correlated with Nugent score, whereas *G. vaginalis*, *Mobiluncus* sp., *A. vaginae* and *M. hominis* were positive correlated (Figure 4). In addition, we observed a strong positive correlation among the loads of *G. vaginalis* and *Mobiluncus* sp. and a strong negative correlation among quantification results of *Lactobacillus* spp. and the BV-associated bacteria *G. vaginalis* and *Mobiluncus* sp. (Figure 5).

Discussion

Vaginal microbiota under homeostatic conditions is predominantly composed by *Lactobacillus*, however some opportunistic pathogens, like *G. vaginalis*, *A. vaginae*, *Mobiluncus* sp. and *M. hominis* may be present in low numbers [4,6,7,10,32]. *Lactobacillus* plays an important role in the structure of vaginal ecosystem by protecting against non-resident bacteria colonization and overgrowth of potentially pathogenic species [34].

PCR-DGGE approach was employed to characterize the bacterial vaginal community among

BV and non-BV women. Distinct richness profiles were observed for the two patient groups and showed that samples from women with the same health status tended to group. In addition, we observed that bacterial communities of women within the same patient group were heterogeneous. Taken together, these findings suggest that vaginal microbiota have different bacterial composition in BV and non-BV women, although it has a common microbe core in both groups [16,17,19,22,35].

Richness refers to the number of operational taxonomic units (OTU) of microbial communities and is a measure that estimates the number of different kinds of organisms present in a particular area; it does not take into account the abundances of the species or their relative abundance distributions and is a component of biological diversity. Diversity is a measure that estimates the number of different species present in a given microbial community as well as the abundance of each species and is used to quantify the biodiversity of a habitat [36]. Actinobacteria and Firmicutes phyla were chosen because they include the most recognized BV-associated bacteria [17]. *Mobiluncus* sp., *G. vaginalis* and *A. vaginae*, which are part of Actinobacteria phylum, were detected at high frequency in women of both groups (BV and non-BV) by qPCR. Despite this, it was possible differentiate BV and non-BV groups towards species richness and diversity, regarding Actinobacteria phylum. Also, according to qPCR results, *Lactobacillus* spp. was identified in almost all women from BV and non-BV groups, whereas *M. hominis* was less often detected among subjects of both groups. *Lactobacillus* spp. and *M. hominis* are part of the same phylum, Firmicutes, and this fact could explain the diversity result, in which it was not possible differentiate BV and non-BV groups. Recent studies based on sequencing techniques have demonstrated the presence of uncultivable and

fastidious species until then not identified in BV-associated bacterial communities [1,16,17,19,21,37].

qPCR assays were performed in order to quantify main bacteria associated with BV and to assess its involvement in this syndrome. *G. vaginalis*, *A. vaginae*, *M. hominis* and *Mobiluncus* spp. were detected at higher concentrations in vaginal microbiota of BV compared to non-BV women, corroborating the findings of recent studies [3,24,30,33,38]. In contrast, *Lactobacillus* spp. was detected at similar concentrations and frequency in the vaginal microbiota of BV and non-BV women, although the non-BV patient group has presented a higher number of subjects with elevated bacterial loads. According to the literature, discrimination of *Lactobacillus* species by qPCR may inform about a shift on the vaginal population of this genus. *Lactobacillus iners* is a species associated with both healthy and abnormal vaginal microbiota [17,23,33]. Unlike *Lactobacillus crispatus* *L. gasseri* and *L. jensenii*, *L. iners* may be common and abundant in abnormal microbiota, sharing space with BV-marker organisms [3,24,33].

We detected the BV-associated bacteria *A. vaginae* in the vaginal microbiota of both BV and non-BV women, however at different loads. A recent study has investigated the community structure of healthy and BV vaginal microbial ecosystems and detected *A. vaginae* only in BV group [25]. However, other studies that also investigated microbial communities associated with BV showed results that corroborate our findings [23,24].

Comparison of bacterial quantification and Nugent score showed the load of *G. vaginalis*, *A. vaginae*, *Mobiluncus* sp. and *M. hominis* increased with the Nugent score increment. In contrast, *Lactobacillus* spp. quantification decreased along Nugent score increase. Such observation indicates even those species which are not scored by Nugent scoring system, such as *A. vaginae* and *M. hominis*, may be good predictors of BV vaginal microbiota together with the classic BV-associated bacteria *G. vaginalis*. Synergic relationship among *G. vaginalis* and *M. hominis* has been demonstrated in BV women, confirming the complexity of this disease [39].

Correlation among BV-associated organisms detected by Nugent score showed that *G. vaginalis* load enhanced concurrently with the raise of *Mobiluncus* sp. concentration, whereas *Lactobacillus* spp. load declined with increased levels of *G. vaginalis* and *Mobiluncus* spp, indicating an antagonistic relationship. These results reinforce the fact of *G. vaginalis* and *Mobiluncus* sp. are involved in BV pathogenesis,

probably in a synergistic way, and *Lactobacillus* spp. is associated with healthy vaginal microbiota.

Intermediate status has been described by several investigators as women harboring a mixed vaginal microbiota transitioning among healthy and BV [40,41]. In the present study, even though there were a small number of intermediate subjects, their vaginal microbiota structure was more similar to those found in BV women, especially regarding to *A. vaginae*, *G. vaginalis* and *Mobiluncus* sp. quantification results. In a recent study, a logistic regression model based on qPCR was created to identify main BV-associated organisms for BV symptomatic diagnosis and it was demonstrated that women with intermediate microbiota could present a vaginal microbial structure similar to the BV microbiota [23].

Although Nugent score is considered the gold standard for BV diagnosis, false negatives may be pointed out as a weakness of the technique [3]. In addition, this is a subjective method that requires specialized training and is not broadly available among physicians in clinical practice [23]. In this context, real-time quantitative PCR technique stands out as a useful tool for molecular BV diagnosis, being more sensitive and providing quantification information about BV-associated organisms not recognized by Nugent score. Real-time quantitative PCR is a very sensitive, specific and reproducible technique that allows the detection of minimal concentration of bacterial DNA and so providing very reliable results. By using primers or probes designed to detect specific regions of the bacterial genomes, the issue regarding false negatives is dramatically reduced. Additionally, this method eliminates the subjectivity that is a characteristic of the microscopic analysis once the results are not affected by the variability inherent of visual analysis of different technicians [42,43].

Bacteria involved in BV syndrome generally are part of healthy vaginal microbiota and because of that quantification assays are essential to improve diagnosis [30,33,42,43]. Recently, multiplex qPCR schemes have been developed to detect and quantify multiple BV-associated organisms with improved accuracy [23,24,44].

Quantitative culture independent approaches for detecting BV are more labor-intensive and costly than Nugent score and Amsel's criteria, however they are reproducible, technician independent, objective, accurate, detect fastidious or uncultivated bacteria and have high specificity. Whereas BV is present worldwide, lowering costs of culture-independent methods is needed to complement clinical diagnosis

and possibly replace the Nugent score system in the future. In addition, regional studies that assess the complexity of vaginal microbial communities may contribute to identification and tracking of the bacterial core associated with BV, which would improve clinical management of patients.

In summary, this study showed that vaginal microbiota is a complex microbial community and have different bacterial composition in BV and non-BV women, although there is a common microbial core in both groups. These facts highlight the importance of the researches focused on elucidating the etiology and pathogenesis of this disease. In addition, we assessed five of the main microorganisms involved on BV pathogenesis and we demonstrated that qPCR technique is a useful and reliable tool for bacteria quantification and an alternative for BV diagnosis once it is capable to detect microorganisms associated with BV that are not identified by the Nugent score system. Also, qPCR can be employed on the clinical practice to monitor BV-associated bacteria dynamic changes during the follow-up of patients and evaluate the treatment effectiveness. Overall, culture-independent molecular approaches may be applied for both clinical diagnostic and research purposes and could give insights about BV etiology and pathogenesis.

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Authors' Contribution

Oliveira, L.M.A: experimentation, data analysis, manuscript edition, Diniz, C.G: experimental design, data analysis, manuscript edition, Fernandes, A.A.S: experimentation under supervision, Souza-Sotte, D.M.K: experimentation, data analysis, Freitas, M.C.R: experimentation, data analysis, Machado, A.B.F: experimentation, data analysis, manuscript edition, Silva, V.L: experimental design, data analysis, manuscript edition, supervision.

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