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Research paper

Identification of vaginal bacteria diversity and it's association with clinically diagnosed bacterial vaginosis by denaturing gradient gel electrophoresis and correspondence analysis

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ABSTRACT

Bacterial vaginosis (BV) is a common complex associated with numerous adverse health outcomes, affecting women of different ages throughout the world. The etiology of BV remains poorly understood due to the difficulty of establishing a molecular genetic criterion to recognize the vaginal microbiota of BV-positive women from that of the normal women. We used techniques of broad-range PCR-DGGE and gel imaging analysis system cooperated with 16S rRNA gene sequencing and statistical analysis to investigate the community structure of the healthy and BV-affected vaginal microbial ecosystems. The community of vaginal bacteria detected in subjects with BV was far more luxuriant and diverse than in subjects without BV. The mean number of microbial species in 128 BV-positive women was nearly two times greater than in 68 subjects without BV(4.05 ± 1.96 versus 2.59 ± 1.14). Our sequencing efforts yielded many novel phylotypes (198 of our sequences represented 59 species), including several novel BV-associated bacteria (BVAB) and many belonging to opportunistic infections, which remain inexplicable for their roles in determining the health condition of vaginal microflora. This study identifies *Algoriphagus aquatilis*, *Atopobium vaginae*, *Burkholderia fungorum*, *Megasphaera genomosp* species as indicators to BV and subjects with BV harbor particularly taxon-rich and diverse bacterial communities. Maybe *Bifidobacterium*, *Staphylococcus* or even more alien species are commensal creatures in normal vaginal microbiota.

1. Introduction

Bacterial vaginosis (BV) is a common vaginal disorder characterized by an alteration of the normal vaginal bacterial microbiota to a heterogeneous state containing a complex population of anaerobic and microaerophilic organisms. BV is associated with sexually transmitted infections and adverse pregnancy outcomes such as low birth weight of foetus, premature birth, neonatal death and secondary infection after delivery and is also the risk factor for premature rupture of fetal membranes (PROM) (Goldenberg et al., 2008). Antibiotics treatment of the condition used to failure due to the uncertain variation of BV-associated bacteria (BVAB) (Beigi et al., 2004; Ferris et al., 2004). Many BV associated bacteria are resistant to culture and (or) difficult to detect by traditional methods, which thwart the exploration of the pathogenesis of BV. So far, there is a lack of effective prevention and treatment measures.

According to Nugent's classification (Nugent et al., 1991), a score ranged from 7 to 10 is considered as BV, a clinical condition dominated by the morphological identification of Gram-negative and-positive bacteria, without evidence of *Lactobacillus* morphotypes. In contrast, the score from 0 to 3 is considered an undisturbed vaginal microflora dominated by the *Lactobacillus* genus, identified as the principal Gram-positive rods bacteria.

In general, race (nationality) (Ness et al., 2003; Uscher-Pines et al., 2009), behavioral habits (Bradshaw et al., 2013), personal hygiene status (Brotman et al., 2008), and so on have been considered as causes of BV. Although there is agreement in the literature that no single agent likely causes BV, there is no consensus about what constitutes a pathogenic bacterial community in this syndrome. Comprehensive cultivation-independent comparisons of the vaginal bacterial communities between subjects with and without clinically defined BV have been rare.

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With the development and application of culture-independent nucleic acid amplification test (NAAT) in recent years, it is possible to explore the structure and dynamic shift of microbiota (Larin et al., 2007; Burton and Reid, 2002; Zozaya-Hinchliffe et al., 2010; Ravel et al., 2011). Molecular methods have identified in the vagina of healthy, nonpregnant women the *Lactobacillus* genus living with a spectrum of bacteria including *Gardnerella*, *Atopobium*, *Eggerthella*, *Megasphaera*, *Leptotrichia*, *Prevotella*, *Enterococcus*, *Bifidobacterium*, *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Bacterioides*, *Mycoplasma*, *Escherichia*, *Peptostreptococcus*, *Ureaplasma*, *Veillonela*, and *Candida* species (Zhou et al., 2004; Ling et al., 2013; Hyman et al., 2005; Vitali et al., 2007; Nam et al., 2007). However, at present the spectrum of bacterial species that are correspondent to clinical diagnosis, i.e. the Nugent's score is not well defined.

In this study, we investigated the composition of BVAB in countryside women of reproductive age. Women with and without BV was diagnosed by Nugent's criteria and recruited as the research subject and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and DNA sequencing techniques were used to analysis the genetic information of 16S rRNA genes (16S rDNA) of vaginal bacteria. The sequence information of 16S rDNA of every detected bacterium was identified with gene bank data. In addition, DGGE Gel Image System was used to calculate the relative abundance of each bacterial species to the whole microbial population of one sample (vaginal swab). Eventually, appropriate statistical method was used to analyze the relationship between BV and bacterial composition, as well as the relationship between BV and other risk factors.

2. Materials & methods

2.1. Ethics statement

All participants enrolled in this study signed written informed consent to their participation. The study protocol and consent form were approved by the ethic review board at Institute of Basic Medicine of Shandong Academy of Medical Sciences.

2.2. Study subjects

196 asymptomatic or symptomatic premenopausal women with a median age of 36 (ranges 18–48) were enrolled from Qilu hospital, Shandong University. 128 women were diagnosed as BV due to their Nugent scores ranging from 7 to 10, whereas 68 were defined as normal that having scores of 0–3. In order to confirm the outcome of Nugent scores criterion, a commercially available test kit (supplied by Sciarray Biotech Co., ShenZhen city, China) was used. This test kit is based on the principle that lactic acid and hydroperoxide are poorly produced by most BV-related organisms and the pH value of vaginal leakage is dramatically different between BV(usually pH ≥ 4.5) and normal(usually pH < 4.5) women. All the women were in good general health and were not undergoing current antibiotic treatment. After thoroughly wiping, the swabs were collected from the posterior fornix of the vaginal tract from 196 women with and without BV, packaged and placed at −40 °C for bacterial DNA extraction and subsequent experiment.

2.3. Extraction of bacterial DNA from vaginal swabs

It is so pivotal for sensitively detecting all the ingredients of the vaginal biota that we adopted an effective DNA extraction method. Briefly, swabs were vigorously agitated in 1 mL of physiological saline solution to disperse cells into a liquid phase. The cells were precipitated in Eppendorf tubes by centrifugation at 10,000*g* for 5 min, washed by re-suspending cells in PBS(pH = 6.8) and centrifuged again at 13,000 g for 3 min. The sediments were re-suspended in 200 μL lysis matrix (mainly consisted of SDS, proteinase K), and the mixture were incubated for 1–2 h in a water bath at 55 °C. The sample was vortexed for 10 s and boiled at 100 °C for 8 min and was vortexed again for 10 s and centrifuged at 13,000*g* for 3 min. The supernatant containing DNA template was stored at −20 °C or used directly for PCR process.

2.4. PCR amplification of the DNA samples

The amplification reactions of the DNA sample were carried out in single 0.2 mL PCR tubes (RNase/DNase/pyrogen free) using a thermocycler (Bio-Rad Laboratories). Each PCR reaction system consisted of 5.0 μL of 10 × buffer (No $MgCl_2$, 10 mM Tris-HCl, and 50 mM KCl), 2.5 μL of $MgCl_2$ (50 mM), 0.5 μL dNTPs (10 mM each), 1.25 μL of glycerol (80%) (Invitrogen™, Life Technologies), 4.0 μL of Bovine serum albumin (BSA) (10 mg/mL) (Invitrogen™, Life Technologies), 50pmoles/μL of primer HDA-1 GC (5′-CGC CCG GGG CGCGCC CCG GGC GGG GCG GGG GCA CGG GGGGAC TCC TAC GGG AGG CAG CAG-3′) and primer HDA-2 (5′-GTA TTACCG CGG CTG CTG GCA-3′) (Invitrogen™, Life Technologies), 0.2 μL of Platinum® Taq DNA polymerase (5 U/μL) (Invitrogen™, Life Technologies), 2.0 μL of the DNA sample, and sterile water to a volume of 50 μL. PCR amplification involved an initial DNA denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °Cfor 30 s, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min, which was followed by a final extension at 72 °C for 7 min. To confirm amplicon production, the mixture (5 μL PCR product and 2 μL of loading buffer) was analyzed by electrophoresis (Bio-Rad Laboratories) using 1.5% Ultrapure™ Agarose (Invitrogen, Life Technologies) gels at 100 V for 45 min, followed by staining with 1% solution of ethidium bromide (50 μL/L). Gels were visualized and recorded by ultraviolet transillumination.

2.5. DGGE (denaturing gradient gel electrophoresis)

Preparation of gel gradients and electrophoresis was carried out according to the manufacturer's instructions for the D-Code™ Universal Mutation Detection System (Bio-Rad Laboratories). The concentrations of the polyacrylamide, gradient denaturant and Tris-acetate buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA [pH 8.0],TAE) were 8%, 30%–60% (7 M urea and 40% deionized formamide were considered to be 100% denaturant), and 50×, respectively. 10 μL of amplified PCR products were mixed with 20 μL of 2 × loading buffer (0.25 mL of bromophenol blue [2%, Sigma], 0.25 mL of xylene cyanol [2%, Sigma], 7 mL of glycerol, and 2.5 mL of distilled H_2O) and loaded into the wells.

Gels were run at 130 V and 65 °C for 3.5–4.0 h in 1 × TAE until the second dye front (xylene cyanol) approached the end of the gel. After electrophoresis, gels were removed and allowed to cool under flowing tap water before the removal of the glass-plate sandwich. The gel was visualized by silver staining and the gel images were analyzed and calculated using a gel imaging and analysis system (chemID™, Saizhi Co, BeiJing city, China). Densitometric measurement of each bands were performed by ChampGel Image System, version 3.0 (Saizhi Co, BeiJing City, China) to generate the ratio of the peak height of a potential band to the sum of the peak heights in the banding profile of every electrophoretic lane, Which corresponded to the relative amount(proportion) of one microbial species to the sum of species in a certain sample.

2.6. Band excision from denaturing gradient gels

DGGE fragment bands were excised from the gels with a sterile scalpel and placed into single 1 mL Eppendorf tubes. Cut bands were washed in 1 × PCR buffer and incubated in 35 μL of the same buffer overnight at 4 °C.

2.7. PCR re-amplification of the cut bands

5 μL of the buffer solution containing fragments from the previously excised bands were used as template for PCR re-amplification. This was conducted using the same PCR mixture to a total volume of 50 μL and eubacterial primers HDA-2 and HDA-1 without the GC clamp. The amplification, annealing and extension conditions were similar to described above.

2.8. Purification of the re-amplified PCR product

The double stranded DNA fragments from PCR re-amplification were purified using EasyPure PCR Purification Kit protocol (TransGen Biotech, Beijing, China). Briefly, 5 volumes of resuspension buffer (RB) was added to 1 vol of the PCR re-amplified sample and mixed in a microcentrifuge tube. This was placed in a spin column with a 2 mL collection tube and centrifuged for 60 s. The flow-through was discarded and the DNA content in the column was washed with 0.75 mL wash buffer(WB) and centrifuged for 60 s twice. The flow-through was discarded and the spin column was placed in a clean 1.5 mL microcentrifuge tube. The DNA was eluted by adding 50 μL elution buffer (EB), left to stand for 5–10 min and centrifuged. The 50 μL flow-through contained the purified DNA.

2.9. Sequencing analysis

Purified DNA samples were sequenced with a BigDye cycle sequencing ready reaction kit (Applied Biosystems). Sequencing reactions were undertaken with an Applied Biosystems 3100 genetic analyzer (Applied Biosystems). Sequences were compared with known sequences by using the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/), and the nearest-neighbor bacterial sequences were aligned with sample sequences by using Vector NTI Advance™ Version 9.0 (Invitrogen, USA). A phylogenetic tree was inferred by the neighbor-joining method with the Phylip software packages.

2.10. Quantification and statistical analysis

2.10.1. Common quantification and statistics

The relative proportion of each species to the whole microbial population in each sample (vaginal swab) was automatically calculated using the ChampGel Image System (chemID™, Saizhi Co, BeiJing city, China) as described above. Differences in the mean number or proportion of taxa between BV(+) and BV(−) women were assessed using the *t*-test for independent two samples. Comparisons of the prevalence rate of *Lactobacillus* or the other species between the two groups were measured with χ^2 test or Fisher's exact probability.

2.10.2. Correspondence analysis

Correspondence analysis is used primarily to identify the correlations among several categorical variables. When the number of categorical variables, or the number of variable values of one or more categorical variables is very large, the commonly used statistical methods such as χ^2 test or multinomial Logistic model is no longer applicable in that these statistical methods can not accurately explain the real connection between every two categorical variables. At this point, the correspondence analysis can solve the above problem. The number of dimensions correspondence analysis can extract equals to the minimum number of studied categorical values minus 1. In this study, we use correspondence analysis to identify the relationship between BV prevalence that having two status and the detected bacteria that having 59 species for the whole participants. If the BV prevalence simply divided into BV positive and the normal group, the corresponding analysis can only extract $2 - 1 = 1$ dimension, which is not in a position for the construction of a corresponding graph. Therefore, the BV positive group should be sub-classified into pure BV and BV & fungal infection group, so that the BV prevalence variables have the total of 3 groups to meet the least criterion that the minimum number of dimensions is equal to 2, thus to build the corresponding graph. Normal group was not sub-classified because the proportion of fungal infection is very small in this study. SPSS software (version 15.0) was used to accomplish all statistic analysis and tests of significance were two-sided with *P* values of <0.05 were considered to indicate statistical significance.

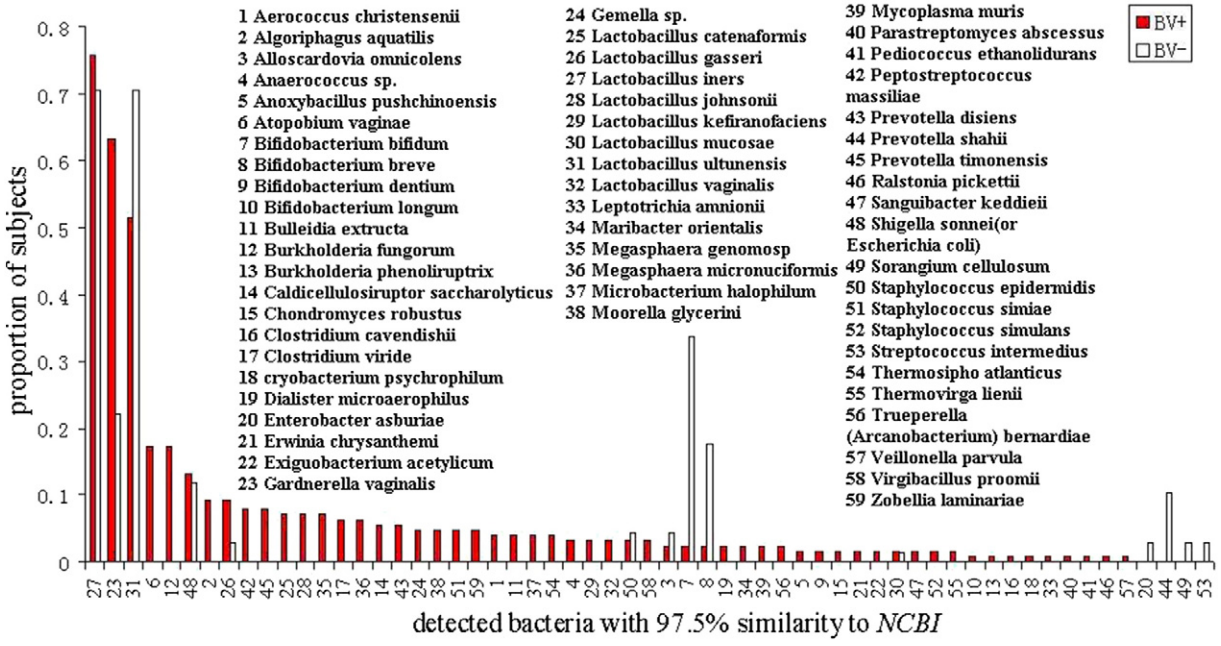

Fig. 1. Prevalence of every detected bacterial species among women with and without bacterial vaginosis. The above list denotes species-level identification with a 97.5% similarity to NCBI taxonomy. The representative strains for every detected species are listed in Table S1.

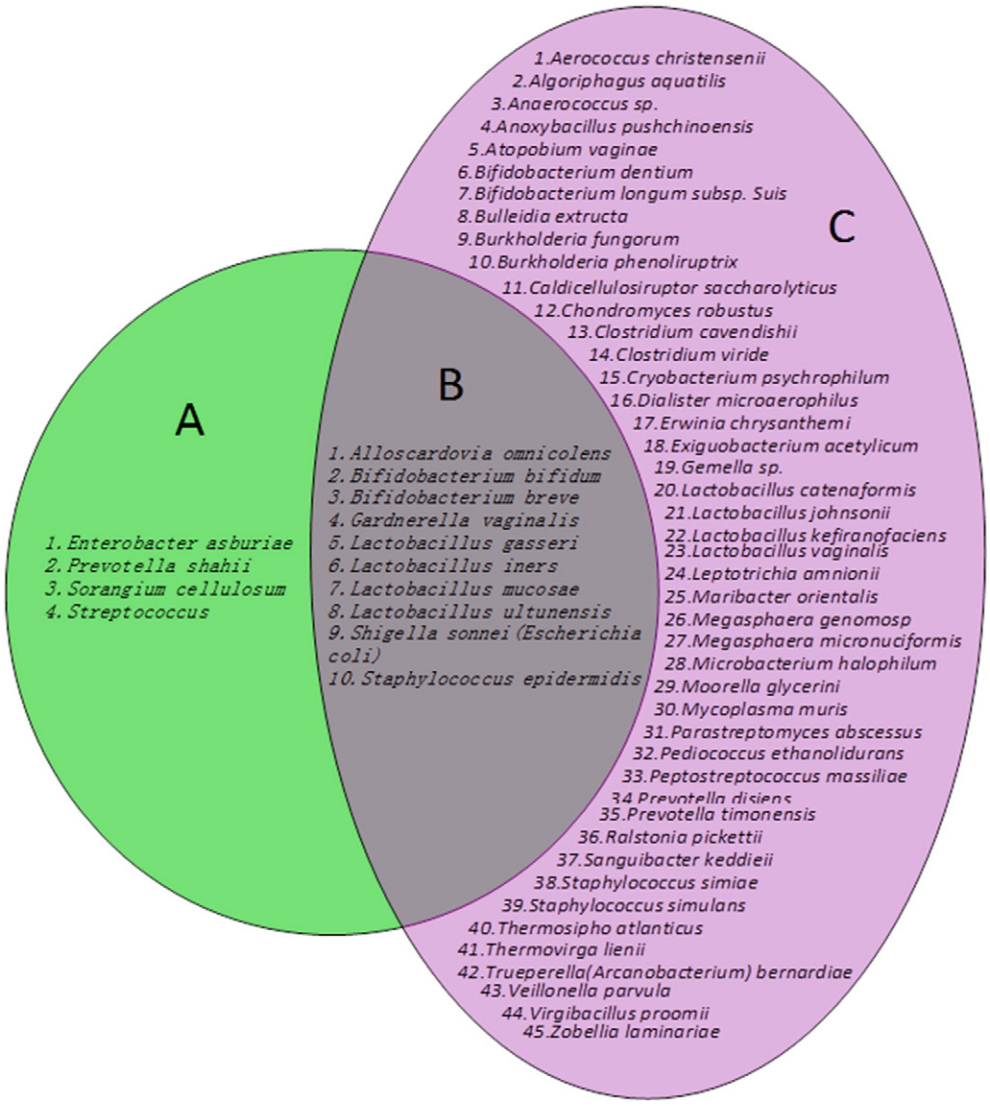

Fig. 2. Bacterial taxa found only in normal women (A), only in BV-positive women (C) and in both norman and BV-positive women (B).

3. Results

3.1. Bacterial species detected in BV positive and normal women

As shown in Figs. 1 and 2, we obtained 198 different DGGE bands from the 196 women, representing 59 species of bacteria based on the nucleotide sequences of the 16S rRNA gene of every DGGE band. A total number of 14 bacteria were found in the normal group and 55 bacteria found in BV group, whereas there were 10 species including *Lactobacillus iners* and *Lactobacillus gasseri*, which are the common species in healthy women found both in the BV and normal women. The species settled in vagina of BV and normal women are only distantly related to each other and to some known species (details were demonstrated in Fig. S1). The genera of *Bifidobacterium bifidum* and *Bifidobacterium breve*, which were commonly considered to harbor only in the mammalian intestinal tract, were found to exist in vaginal microbiota of BV or normal women. Table 1 shows the difference of bacterial load, bacterial diversity and vaginal pH value between BV and normal group. The average bacterial load (1069.34 ± 113.38 versus 317.50 ± 59.09) that represents the volume of total bacteria, average number of bacterial species (4.05 ± 1.96 versus 2.59 ± 1.14) and vaginal pH value (3.79 ±

Table 1
Comparison of bacterial load, mean bacterial number and vaginal pH between normal and BV-positive group.

Compared item	Nugent diagnosis	*n*	$\bar{x} \pm s$	*t*	*P*
Bacterial load (IOD*)	Normal	68	317.50 ± 59.09	/	/
	BV	128	1069.34 ± 113.38	5.108	<0.001
OTUs▲ per woman	Normal	68	2.59 ± 1.14	/	/
	BV	128	4.05 ± 1.96	5.660	<0.001
Vaginal pH	Normal	68	3.56 ± 0.41	/	/
	BV	128	3.79 ± 0.62	2.707	0.007

* integrated OD value; ▲ operational taxonomic units.

0.62 versus 3.56 ± 0.41) were higher in BV positive than in normal group. In addition, there were two 16S rDNA sequences of BV-associated bacteria (BVAB) that having <97.5% homology compared with the standard database in *NCBI*. They were named as *BVAB*1 that having the utmost 96.835% homology to *Lactobacillus ultunensis* and *BVAB*2 that having the maximum 86.184% homology to *Mycoplasma muris*.

3.2. A comparison of bacteria prevalence and relative proportion between normal and BV positive group

The prevalence rates of the 59 kinds of bacteria in normal and BV groups were estimated, while the relative proportion of each bacterial species among the entire microbial population in every woman vaginal microenvironment was also determined by gel image analysis system.

Table 2
Bacteria prevalence and relative abundance in the normal and BV-positive group.

	BV-positive (n = 128)		Normal (n = 68)		Prevalence comparison		Abundance comparison		
Detected bacteria	Prevalence %	Mean abundance %	Prevalence %	Mean abundance %	χ^2	*P*	Normal-BV	*t*	*P*
1. *Aerococcus hristensenii*	3.9063	0.5588	0.0000	0.0000	/▲	0.1660	−0.5588	−1.5335	0.1268
2. *Algoriphagus aquatilis*	9.3750	1.9472	0.0000	0.0000	/	0.009*	−1.9472	−2.2915	0.0230*
3. *Alloscardovia omnicolens*	2.3438	0.4447	4.4118	1.2496	/	0.420	0.8049	1.1203	0.2640
4. *Anaerococcus* sp.	3.1250	0.6764	0.0000	0.0000	/	0.300	−0.6764	−1.4131	0.1592
5. *Anoxybacillus pushchinoensis*	1.5625	0.1985	0.0000	0.0000	/	0.545	−0.1985	−0.8943	0.3723
6. *Atopobium vaginae*	17.1875	3.3221	0.0000	0.0000	13.165	<0.001*	−0.5588	−3.1830	0.0017*
7. *Bifidobacterium bifidum*	2.3438	0.4317	33.8235	7.0160	38.249	<0.001*	6.5842	4.8803	<0.001*
8. *Bifidobacterium breve*	2.3438	0.3969	17.6471	2.7847	14.716	<0.001*	2.3878	2.9416	0.0037*
9. *Bifidobacterium entium*	1.5625	0.3699	0.0000	0.0000	/	0.545	−0.3699	-1.0331	0.3029
10. *Bifidobacterium longum* subsp. *suis*	0.7813	0.2727	0.0000	0.0000	/	1.000	−0.2727	−0.7280	0.4675
11. *Bulleidia extructa*	3.9063	0.4112	0.0000	0.0000	/	0.166	−0.4112	−1.5861	0.1143
12. *Burkholderia fungorum*	17.1875	3.3981	0.0000	0.0000	13.165	<0.001*	−3.3981	−3.3253	0.0011*
13. *Burkholderia phenoliruptrix*	0.7813	0.0922	0.0000	0.0000	/	1.000	−0.0922	−0.7280	0.4675
14. *Caldicellulosiruptor saccharolyticus*	5.4688	1.3023	0.0000	0.0000	/	0.098	−1.3023	−1.7111	0.0887
15. *Chondromyces obustus*	1.5625	0.0528	0.0000	0.0000	/	0.545	−0.0528	−0.9609	0.3378
16. *Clostridium avendishii*	0.7813	0.0500	0.0000	0.0000	/	1.000	−0.0500	−0.7280	0.4675
17. *Clostridium viride*	6.2500	1.0065	0.0000	0.0000	/	0.052	−1.0065	−1.7419	0.0831
18.*cryobacterium psychrophilum*	0.7813	0.0176	0.0000	0.0000	/	1.000	−0.0176	−0.7280	0.4675
19. *Dialister croaerophilus*	2.3438	0.3346	0.0000	0.0000	/	0.553	−0.3346	−1.0227	0.3077
20. *Enterobacter asburiae*	0.0000	0.0000	2.9412	0.2830	/	0.119	0.2830	1.9568	0.0518
21. *Erwinia chrysanthemi*	1.5625	0.1836	0.0000	0.0000	/	0.545	−0.1836	−0.9228	0.3573
22. *Exiguobacterium acetylicum*	1.5625	0.0291	0.0000	0.0000	/	0.545	−0.0291	−1.0217	0.3082
23. *Gardnerella vaginalis*	63.2813	16.5287	22.0588	8.2078	30.197	<0.001*	−8.3209	−2.7929	0.0057*
24. *Gemella* sp.	4.6875	0.5549	0.0000	0.0000	/	0.094	−0.5549	−1.6258	0.1056
25. *Lactobacillus catenaformis*	7.0313	1.6728	0.0000	0.0000	/	0.029*	−1.6728	−2.0790	0.0389*
26. *Lactobacillus gasseri*	9.3750	2.4013	2.9412	2.0279	/	0.144	−0.3734	−0.2280	0.8199
27. *Lactobacillus iners*	75.7813	24.9039	70.5882	37.1071	0.622	0.495	12.2032	2.6870	0.0078*
28. *Lactobacillus johnsonii*	7.0313	2.1192	0.0000	0.0000	/	0.029*	−2.1192	−1.7550	0.0808
29. *Lactobacillus kefiranofaciens*	3.1250	0.3192	0.0000	0.0000	/	0.300	−0.3192	−1.3394	0.1820
30. *Lactobacillus mucosae*	1.5625	0.3107	1.4706	0.4417	/	1.000	0.1310	0.2973	0.7666
31. *Lactobacillus ultunensis*	51.5625	19.0862	70.5882	33.2635	6.606	0.010*	14.1772	3.3739	0.0009*
32. *Lactobacillus vaginalis*	3.1250	0.4533	0.0000	0.0000	/	0.300	−0.4533	−1.1477	0.2525
33. *Leptotrichia amnionii*	0.7813	0.2095	0.0000	0.0000	/	1.000	−0.2095	−0.7280	0.4675
34. *Maribacter orientalis*	2.3438	0.2173	0.0000	0.0000	/	0.553	−0.2173	−1.1971	0.2327
35. *Megasphaera enomosp*	7.0313	0.9111	0.0000	0.0000	/	0.029*	−0.9111	−1.8009	0.0733
36. *Megasphaera micronuciformis*	6.2500	2.7784	0.0000	0.0000	/	0.052	−2.7784	−1.8717	0.0628
37. *Microbacterium halophilum*	3.9063	0.1722	0.0000	0.0000	/	0.166	−0.1722	−1.3172	0.1893
38. *Moorella glycerini*	4.6875	0.4839	0.0000	0.0000	/	0.094	−0.4839	−1.6889	0.0928
39. *Mycoplasma muris*	2.3438	0.3333	0.0000	0.0000	/	0.553	−0.3333	−1.0840	0.2797
40. *Parastreptomyces abscessus*	0.7813	0.0725	0.0000	0.0000	/	1.000	−0.0725	−0.7280	0.4675
41. *Pediococcus ethanolidurans*	0.7813	0.1820	0.0000	0.0000	/	1.000	−0.1820	−0.7280	0.4675
42. *Peptostreptococcus massiliae*	7.8125	0.6302	0.0000	0.0000	/	0.016*	−0.6302	−2.2417	0.0261*
43. *Prevotella disiens*	5.4688	1.5567	0.0000	0.0000	/	0.098	−1.5567	−1.5318	0.1272
44. *Prevotella shahii*	0.0000	0.0000	10.2941	0.7575	/	<0.001*	0.7575	3.5884	0.0004*
45. *Prevotella timonensis*	7.8125	0.6233	0.0000	0.0000	/	0.016*	−0.6233	−1.7980	0.1268
46. *Ralstonia pickettii*	0.7813	0.0376	0.0000	0.0000	/	1.000	−0.0376	−0.7280	0.0230*
47. *Sanguibacter keddieii*	1.5625	0.2156	0.0000	0.0000	/	0.545	−0.2156	−1.0134	0.2640
48. *Shigella sonnei* (or *Escherichia coli*)	13.2813	4.0245	11.7647	3.6161	0.092	0.826	−0.4085	−0.2152	0.1592
49. *Sorangium ellulosum*	0.0000	0.0000	2.9412	0.4174	/	0.119	0.4174	1.8798	0.3723
50. *Staphylococcus epidermidis*	3.1250	0.3203	4.4118	1.7561	/	0.695	1.4358	1.7600	0.0017*
51. *Staphylococcus imiae*	4.6875	1.2150	0.0000	0.0000	/	0.094	−1.2150	−1.5958	<0.001*
52. *Staphylococcus mulans*	1.5625	0.1520	0.0000	0.0000	/	0.545	−0.1520	−1.0152	0.0037*
53. *Streptococcus intermedius*	0.0000	0.0000	2.9412	1.0717	/	0.119	1.0717	1.8095	0.3029
54. *Thermosipho atlanticus*	3.9063	0.3468	0.0000	0.0000	/	0.166	−0.3468	−1.4255	0.4675
55. *Thermovirga lienii*	1.5625	0.3191	0.0000	0.0000	/	0.545	−0.3191	−0.8360	0.1143
56. *Trueperella* (*Arcanobacterium*) *bernardiae*	2.3438	0.2817	0.0000	0.0000	/	0.553	−0.2817	−1.2157	0.0011*
57. *Veillonella parvula*	0.7813	0.0392	0.0000	0.0000	/	1.000	−0.0392	−0.7280	0.4675
58. *Virgibacillus proomii*	3.1250	0.1420	0.0000	0.0000	/	0.300	−0.1420	−1.3901	0.0887
59. *Zobellia laminariae*	4.6875	0.8889	0.0000	0.0000	/	0.094	−0.8889	−1.3775	0.3378
Total *lactobacillus*	92.9688	51.2667	95.5882	72.8402	/	0.548	21.5736	4.440	<0.001*

▲ χ^2 cannot be calculated, whereas we got a *P* value using Fisher exact probability method.
* The difference was significant at the 0.05 level of significance.

As shown in Table 2, the genus of *Lactobacillus* were dominant both in normal and BV groups, with a total *Lactobacillus* prevalence of 92.97% and 95.59% respectively and the difference was not statistically significant (Fisher's exact $P = 0.548$), but the proportion of total *Lactobacillus* was significantly higher in normal group than in BV group (72.84% vs 51.27%, $t = 4.440$, $P < 0.001$). *Lactobacillus iners* represented the most common species in patient group(75.78%) and normal group(70.59%), also having no significant difference, but the relative proportion in normal group is significantly higher than that in BV group (37.11% vs 24.90%, $P = 0.0078$). Both the prevalence rate and the relative proportion of *Lactobacillus ultunensis* were significantly higher in normal group than in the patient group. Meanwhile, women from normal group had both higher prevalence rates and higher relative proportions for *Bifidobacterium bifidum* and *Bifidobacterium breve* compared with women from the BV group which is a proof that *Bifidobacterium* was also normal vaginal bacteria. *Gardnerella vaginalis*, a marker of bacterial vaginosis, was a prominent species in the BV group in terms of the prevalence rate and relative proportion. We did not detect *Atopobium vaginae* in normal group, but it presented in BV group with a positive rate of 17.19% and a mean relative proportion of 3.32%.

2 kinds of *Prevotella* species were detected, with *Prevotella shahii* found in the normal group and *Prevotella timonensis* found in BV group. But there were 2 kinds of *Staphylococcus* (*Staphylococcus simiae* and *Staphylococcus simulans*) to be discovered only in the BV group. *Peptostreptococcus massiliae* only inhabited in the BV group, of which the detection rate and the relative proportion were 7.81% and 0.63% respectively. The following genera and their correlation to BV have been not or rarely reported: (1) *Burkholderia fungorum* was not detected in the normal group, the positive rate and relative proportion of which in BV positive group were 17.19% and 3.40% respectively. In addition, *Megasphaera genomosp*, *Megasphaera micronuciformis*, *Algoriphagus aquatilis* and *Arcanobacterium bernardiae* was exclusively detected in women of BV.

(2) The infection rate of *Escherichia coli*, which belongs to genus of intestinal *Bacillus*, was no <10% either in BV or in normal group, and the difference was not statistically significant ($P = 0.826$).

3.3. Correspondence analysis

Fig. 3 showed the result of correspondence analysis that was performed based on the data of mean abundance (relative proportion) of bacterial species detected by DGGE and DNA sequencing method. As shown in Fig. 3, there had good discrimination in dimension 1 and dimension 2 for the normal, BV and BV & fungal vaginitis (FV) group, which located in I, III and IV quadrant, respectively. In the first quadrant, there were species of 3, 7, 8, 20, 27, 31, 44, 49, 50 that associated with the normal group. BV-associated bacteria is 2, 6, 10, 12, 13, 14, 15, 16, 17, 35, 54, 59 and species of 1, 4, 5, 9, 19, 22, 29, 48 had relation to condition of BV & FV affection.

3.4. The comprehensive judgment of BV-related bacteria

As mentioned above, we used comparison of prevalence (Table 2), comparison of relative proportion (Table 2) and correspondence analysis to identify the correlations of every bacterial species to health conditions. The results were shown in Table 3.

Whether by comparison of prevalence or comparison of relative proportion or correspondence analysis, we discovered a strong connection between normal women and species 7, 8, 27, 31, 50 that represented *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Lactobacillus iners*, *Lactobacillus ultunensis*, *Staphylococcus epidermidis*, respectively. Meanwhile, bacteria 2, 6, 12, 35, which corresponded to *Algoriphagus aquatilis*, *Atopobium vaginae*, *Burkholderia fungorum*, *Megasphaera genomosp* had relation to BV. It can be seen from Fig. 3 that *Gardnerella vaginalis* (represented by 23) located nearly at the coordinate origin which interpreted the fact that *Gardnerella vaginalis* is not specific to BV or to the normal condition.

4. Discussion

In this study, the broad-range PCR using a pair of primers commonly specific to the conservative region of bacteria rRNA genes was conducted. The PCR products were analyzed by DGGE, and DNA molecules in each DGGE bands were sequenced to obtain information of bacterial taxonomy. The relative abundance of every bacterium was determined according to the comparison of absorbance of each DGGE band with that of all bands in one DGGE profile that theoretically represents the whole population of a certain vaginal microflora. Accordingly, we found 1–8 DGGE bands in each of the 68 normal women and 2–13 DGGE bands in each of the 128 BV-positive women. Therefore, only no >13 sequencing for each BV-positive vaginal fluid and no >8 sequencing for each normal specimen are necessary to calculate the relative proportion of every detected bacteria. By comparison, the commonly used clone library method should perform at least one hundred or more sequencing for each specimen in order to prevent the omission of bacteria of very low concentration level(i.e. <1%). PCR-DGGE sequencing method employed in this study has the advantage of faster performance and cheaper expenditure compared with previously reported methods, such as clone library. So specimens derived from an even larger sample can be analyzed, such as the sample size in this study was 196, far larger than the similar studies in the past.

Like many reports (Fredricks et al., 2005; Verhelst et al., 2005; Verstraelen et al., 2004), our PCR-DGGE method detected many more kinds of bacteria compared with the culture-based method and as many kinds of bacteria as clone library analysis. Either for normal or

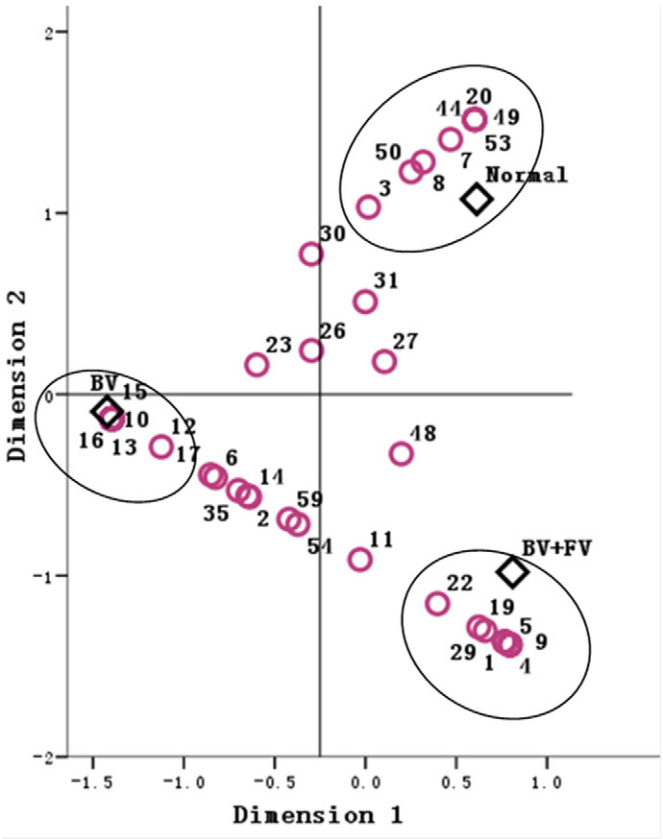

Fig. 3. Correspondence analysis of intravaginal microbial flora based on the relative abundance of bacteria. ◇ BV diagnosis by the conventional methods (Nugent's criteria); ○ detected bacterial species by 16S rRNA gene sequencing; each digital number denotes one certain microbial species as described in Table 2.

Table 3
Summary of the association between bacteria and the clinical diagnosis of BV.

Comparison of prevalence and relative proportion		Correspondence analysis			Comprehensive results	
Normal	BV	Normal	BV	BV & FV	Normal	BV
7, 8, 27, 31, 44, 50	2, 6, 12, 23, 25, 35, 42, 45, 46, 51, 52, 56	3, 7, 8, 20, 27, 31, 44, 49, 50, 53	2, 6, 10, 12, 13, 14, 15, 16, 17, 35, 54, 59	1, 4, 5, 9, 11, 19, 21, 22, 28, 29, 43, 48	7, 8, 27, 31, 50	2, 6, 12, 35

*The correspondence between digits and microbial species equals to what was described in Table 2.

BV group, the vaginal discharge showed distinctly different microbial communities in terms of the composition of species as well as of the volume of total bacteria. As for the dominant inhabitant, *Lactobacillus* spp. boasted an equal prevalence among women of the 2 groups but a higher concentration in the normal woman than in BV-positive women and this species is a key point for understanding the state of intravaginal microflora. Previous reports suggest that *Lactobacillus crispatus*, *Lactobacillus iners*, and other *Lactobacillus* spp. play the pivotal roles for the discrimination between normal and abnormal intravaginal microbial flora (Ferris et al., 2007; Jakobsson and Forsum, 2007). Like the report from Zhou et al., we found that *Lactobacillus iners* was frequently detected as a dominant species (Zhou et al., 2004; Zhou et al., 2007; Zhou et al., 2010; Fredricks, 2011). Kaewsrichan et al. reported that *L. iners* is a dominant part of the vaginal flora when the flora is in a transitional stage between abnormal and normal. Meanwhile, it was reported that *L. crispatus* produce more H_2O_2 and bactericidal agents compared with other *Lactobacilli* so that it is considered as a leading role in maintaining the health of vaginal ecosystem (Kaewsrichan et al., 2006). Unfortunately, we did not found *Lactobacillus crispatus* in the 2 groups. According to our findings, women with *Lactobacillus iners*-dominant and *Lactobacillus crispatus*-absent intravaginal microbial flora, who were presumed to be normal by the conventional methods, may be thought to be at risk of BV and must be monitored carefully.

Other than *Lactobacillus* spp., it is important to note that *Algoriphagus aquatilis*, *Atopobium vaginae* (ananaerobe), *Burkholderia fungorum* and *Megasphaera enomosp* are characteristic to the BV group, though the species have not been detected by the culture-based method. These results correspond with the report (Menard et al., 2008) that *A. vaginae* is associated with BV. Consequently, our results indicate that the relative proportions of these species were key points for the recognition of the normal and BV-positive women.

Culture-based methods other than PCR-DGGE were frequently used to detect *G. vaginalis*, a BV-related facultative anaerobe, while *A. vaginae*, *Burkholderia fungorum*, *Megasphaera enomosp*, and other anaerobes were usually identified by PCR-DGGE. Previous reports confirmed that *G. vaginalis* is not a single, specific species to cause BV (Srinivasan and Fredricks, 2008; Fredricks and Marrazzo, 2005). In this study, we only found a weak linkage between *G. vaginalis* and BV. In addition, our correspondence analysis didn't find that *G vaginalis* is strongly associated with BV or normal condition. In other words, *G. vaginalis* is not an independent risk factor for BV condition.

Bifidobacterium bifidum, *Bifidobacterium breve* and *Escherichia coli* belong to intestinal inhabitants, but they were all detected from vaginal secretion in this study. The possibility of opportunistic infections is likely to be huge, probably due to the poor personal hygiene. *Escherichia coli* was detected with almost equal prevalence and relative abundance between the 2 groups, so it is thought to be just a opportunistic contamination. While the two *Bifidobacterium* had higher abundance and prevalence in the normal than in BV group, it implicates that they may be also potential health maintainers for vaginal microenvironment.

In conclusion, our broad-range PCR-DGGE method and gel imaging analysis system can provide useful information about bacterial communities. In combination with the correspondence analysis, we identified *Algoriphagus aquatilis*, *Atopobium vaginae*, *Burkholderia fungorum*, *Megasphaera genomosp* species that are individually or as a whole specific to the occurrence of bacterial vaginosis. As for the composition of health-maintaining microbial community, the dynamic changes of *Lactobacillus* family are important to monitor the health status of vaginal flora. In this context, most of the seemingly normal women are in fact potentially hazardous to develop into BV. Moreover, we postulate that there has some commensal relationships between *Lactobacillus* genus and other taxa such as *Bifidobacterium*, *Staphylococcus* that has been found in women from China rural area in this study. Compared with conventional examinations, our method will be a helpful tool for accurate diagnosis and treatment. More importantly, our findings give researchers and clinical practitioners more insight into the essential etiology of bacterial vaginosis.

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Author contributions

Qing Xa and Hua Zhang performed most of the experiments; Yutao Diao as the corresponding author designed the study, wrote the manuscript, performed part of the experiments; Hao Li and Lijuan Cheng were responsible for data collection and data entry; Shangwen Sun accomplished the statistical work.

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Potential conflicts of interest

None of the other authors have any conflict of interest to report.

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