Characterisation of the Vaginal Microbiota Using Culturomics and Metagenomics Suggests Transplantation of Gut Microbiota Into the Vagina During Bacterial Vaginosis

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Research

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Abstract

Background: Bacterial vaginosis is a very common vaginal disorder, with a gynaeco-obstetrical impact, but remains poorly understood. Current antibiotic treatment most often fails. Our objective was to exhaustively map the bacterial community present in bacterial vaginosis and normal flora, using not only a molecular approach but also a highly efficient culture approach, culturomics. Vaginal bacterial diversity was evaluated using both strategies for 24 Caucasian French women, including 7 cases of bacterial vaginosis, and 10 rural Senegalese women, including 5 cases of bacterial vaginosis. An additional 16 specimens (three cases of bacterial vaginosis and 13 normal flora) obtained during the follow-up visits of five French women with bacterial vaginosis were analysed using metagenomics.

Results: The combination of culturomics and metagenomics reveals the richness and diversity of vaginal microbiota. Our data demonstrated that compared to healthy women, those with bacterial vaginosis present a shift in vaginal flora. The microbiota of the bacterial vaginosis group can be easily distinguished from that of healthy group by their increased bacterial diversity, abundance of *Bacteroidales* and *Leptotrichiaeceae*, and depletion of *Proteobacteria* and *Lactobacillaceae* species. In addition, a complex of 10 genera was associated with bacterial vaginosis: *Gardnerella, Atopobium, Snethia, Aerococcus, Prevotella, Gemella, Facklamia, Porphyromonas, Mycoplasma,* and *Urinacoccus.* Overall, the microbial population detected in bacterial vaginosis flora was largely similar to the bacterial communities found in gut microbiota. The two approaches showed a diverse vaginal microbiota that was largely non-overlapping, with only 146 common species. Furthermore, culturomics extends the repertoire of human-associated bacteria with the isolation of 27 new bacterial species.

Conclusions: This study provides the most representative topology of the vaginal microbiota of normal flora and bacterial vaginosis and suggests the transplantation of gut microbiota into the vagina during bacterial vaginosis. Several sets of clinically relevant anaerobic bacteria and new species were also isolated from the human vagina, confirming that some previously “uncultivated” species may be cultivated using an appropriate strategy. Exhaustive characterisation of vaginal microbiota will enable a better understanding and management of bacterial vaginosis. Obtaining isolates will allow us to explore the bacterial interactions of the vaginal microbiota and will serve as a basis for the potential development of bacteriotherapy to prevent or treat vaginal dysbiosis.

Background

Bacterial vaginosis is the most prevalent vaginal disorder in pubertal women [1]. It most often causes leucorrhoea with a foul odour and sometimes irritations that lead women to seek gynaecological care [2]. It is associated with the risk of preterm birth, miscarriage, and low birth weight when presenting in pregnant women [3], as well as the risk of pelvic inflammatory diseases [4]. Bacterial vaginosis predisposes women to serious health problems, including the acquisition and transmission of various pathogens such as herpes simplex virus-2 [5], human immunodeficiency virus-1 [6], papillomavirus [7], *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, and *Chlamydia trachomatis* [8, 9]. Depending on the population being studied, its prevalence ranged from 4–75% between asymptomatic women and those with sexually transmitted diseases, respectively [10, 11]. To manage bacterial vaginosis and relieve symptoms, the Center for Disease Control and Prevention recommend treating all patients with clinical manifestations [12] (homogeneous vaginal discharge adhering to the vaginal walls, unpleasant odour, vaginal pH > 4.5 and presence of clue cells [13]). However, up to 50% of affected women seem to have no symptoms and antibiotic therapy often fails with a 50% relapse rate after six months of treatment [14, 15].

A disturbance of the native bacterial flora present in the healthy vaginal tract is widely observed in bacterial vaginosis. Under normal conditions, the healthy vagina of premenopausal women is quantitatively dominated by lactobacilli, which make up approximately 70–90% of all bacteria [16]. Lactobacilli are lactic acid bacteria and some species can produce hydrogen peroxide to protect the vaginal tract and prevent the proliferation of other vaginal microorganisms [17, 18]. Previous studies have shown that during bacterial vaginosis, the vaginal tract flora undergoes significant changes ranging from predominantly *Lactobacillus* flora to colonisation without *Lactobacillus* [19]. The etiology of this gynaecological condition is still unknown and it remains one of the great enigmas around women’s health. To understand whether changes in the vaginal microbiota could be linked with health, the Human Microbiome Project (HMP) considers that it is essential to examine the bacterial communities of the healthy vaginal tract and those of the bacterial vaginosis [20].

In the past, the vaginal bacterial communities have been identified using culture-based methods. Due to the inability to isolate most of the bacterial species present in the vagina, the taxonomic composition of this ecosystem is not well understood. In recent decades,

many studies have focused on the vaginal microbiota using advances in technology such as more efficient molecular techniques (based mostly on 16S rRNA gene sequencing). These have revealed that the vaginal microbiota is more complex than previously suspected. Furthermore, bacterial vaginosis is a dysbiosis. Bacterial vaginosis is typified by the reduction in lactobacilli and the overgrowth or introduction of anaerobic bacteria and Bacterial Vaginosis (BV)-associated bacteria [21] that are also found in the human gut. For a better understanding of this public health problem, it is necessary to use culture methods to isolate and study these BV-associated bacteria currently observed only using molecular methods. This renaissance of culture-based methods has been observed through the development of microbial culturomics, an approach which multiplies the conditions of culture and variation of physicochemical parameters coupled with a rapid identification of bacteria using MALDI-TOF (Matrix Assisted Laser Desorption Ionisation-Time of Flight) mass spectrometry [22, 23].

In this study, using metagenomics targeting the 16S rRNA gene and culturomics, we investigated the vaginal bacterial diversity of normal and bacterial vaginosis floras in French and rural Senegalese women in order to better understand the dysbiosis of vaginal microbiota that occurs during bacterial vaginosis.

Results

Characteristics of the studied population and profile of the vaginal microflora

The present study included 34 women aged between 20 and 50 (28.53±5.74), whose vaginal flora was characterised (Table 1). Ten vaginal specimens were sampled in Senegal from five patients with bacterial vaginosis and five healthy women. Twenty-four specimens were sampled in France: seven specimens from seven patients with bacterial vaginosis and 17 from healthy women. The culture of these 34 vaginal samples enabled us to isolate and identify 340 bacteria from seven phyla, 52 families, and 128 genera (Table S1). In addition, during the follow-up visits in France, 16 other vaginal specimens were sampled from five women had previously been diagnosed with bacterial vaginosis. Of them, three were diagnosed as having bacterial vaginosis and 13 as having normal flora. All 50 vaginal specimens were analysed using metagenomics barcoding the “V3-V4 region” of the 16S rRNA gene. A total of 1,215,586 and 2,946,743 reads were generated from the 15 samples with bacterial vaginosis and from the 35 normal vaginal flora samples, respectively. These sequences correspond to 1,246 OTUs including 1,229 bacteria assigned to 169 genera, 70 families, and nine phyla (Table S2). Only 383 of these OTUs (31%) were classified at the species level. In the bacterial vaginosis group, 46% of these OTUs corresponded to *Gardnerella vaginalis* (26%), *Atopobium vaginae* (12%), and *Lactobacillus iners* (8%), whereas *Lactobacillus crispatus* (15%), *Bacillus simplex* (11%), *Escherichia coli* (10%), *L. iners*, and *G. vaginalis* (7%, of each) represented the major OTUs identified in healthy women. Overall, only *Bifidobacterium scardovii* and *Facklamia ignava* were detected using both metagenomics and culturomics, and only among patients with bacterial vaginosis. In addition, a panel of 15 species (*Acidaminococcus intestini, Acinetobacter baumannii, Alistipes putidinis, Bacteroides cellulosilyticus, Bacteroides fragilis, Bacteroides salyersiae, Enterococcus pallens, Lactobacillus mucosae, Macrococcus caseolyticus, Morganella morganii, Phascolarctobacterium faecium, Pseudoramibacter alactolyticus, Streptococcus australis, Streptococcus urinalis*, and *Trueperella bernardiae*) was only found among normal vaginal flora using these two techniques (Table 1).

As previously described [24–26], according to the dominant OTUs, the vaginal microbiota profiles of our population did not cluster together and formed six community state types (CSTs). Normal vaginal microbiota profiles were assigned onto CST IV A (35%, heterogenous with various aerotolerant species), CST I (18%, *L. crispatus* dominant), CST III (9%, *L. gasseri* dominant), and CST V (3%, *L. iners* dominant), while the vaginal flora from bacterial vaginosis microbiota samples was grouped onto two clusters: CST IVC (21%, dominated by *Gardnerella vaginalis* and *Megasphaera* sp.) and CST IVD (14%, mixture of *Atopobium vaginae, Snethia*, *Peptoniphilus,* and *Prevotella*).

High vaginal microbiota diversity in flora with bacterial vaginosis

A total of 1,194,818 and 2,484,424 reads were generated from the 12 vaginal flora with bacterial vaginosis and the 22 normal samples, respectively. Estimation of α-diversity showed that the vaginal microbiota of women with bacterial vaginosis were richer (ACE 34.8±1.7, Chao-1 29.5±16.5, Table 1) and more diverse (Shannon index 1.9 ± 0.7, Simpson index 0.3 ± 0.2, Figure 1) than those of healthy women (28.7±1.5, 22.5±15.5, 1.3±0.8, and 0.5±0.3, respectively). The bacterial communities were also more abundant during episodes of bacterial vaginosis than in healthy samples (evenness 0.14 in bacterial vaginosis versus 0.09 in normal vaginal flora, p=0.009, unpaired t-test). In addition, the hitherto unknown diversity (unclassified OTUs) was significantly increased in patients with bacterial vaginosis (p=0.03, unpaired t-test, Figure 1).

According to culturomics analysis, β-diversity was higher in the bacterial vaginosis group [115/241 (48%)] than in the group with normal vaginal flora [110/261 (42%)]. Among the patients with bacterial vaginosis, 43% of isolated species (103/241) were not previously known to be present in the human vagina [27], including 14 new bacterial species and four new genera (Table 2 [28–40]). Among healthy women with normal vaginal flora, 46% (119/261) species were not known to be present in the human vagina, including seven new species and two new genera. The hitherto unknown diversity (new species) detected by culturomics increased considerably during bacterial vaginosis (p=0.03, Mann Whitney test, Figure 1).

Dynamic modification of vaginal microbiota during bacterial vaginosis

Given the strong evidence that the structure of the vaginal microbiota differs strikingly between healthy women and those with bacterial vaginosis, we sought to identify differences that occur during this dysbiosis. Metagenomic analysis of samples from women with bacterial vaginosis and those from healthy women generated 1,118,379 and 2,466,547 reads assigned at the species level, respectively. The reads matched with a total of 360 bacterial species (208 species for bacterial vaginosis and 307 for normal vaginal flora), which were classified into nine phyla: *Actinobacteria, Bacteroidetes, Epsilonbacteraeota, Firmicutes, Fusobacteria, Proteobacteria, Synergistetes* (only in the healthy vaginal flora)*, Tenericutes,* and *Verrucomicrobia* (only in bacterial vaginosis). The vaginal microbiota from bacterial vaginosis includes significantly more *Actinobacteria* (67/208 versus 68/307, p=0.01, exact Fischer test) and fewer *Proteobacteria* (16/208 versus 64/307, $p=3.10^{-4}$) than those in healthy flora.

Interestingly, LEfSe analysis revealed that the significant abundance of *Actinobacteria* in women with bacterial vaginosis was due to the increased number of bacteria belonging to *Bifidobacteriaceae*. In addition, the relative abundance of *Leptotrichiaceae* (phylum *Fusobacteria*) and *Bacteroidales* (phylum *Bacteroidetes*) microorganisms were also increased during bacterial vaginosis. In healthy women, three clades were significantly overrepresented: two belonging to *Proteobacteria* (*Enterobacteriaceae*/ *Pseudomonadaceae* and *Sutterellaceae* families) and one clade to *Lactobacillaceae* (Figure 2A). An LDA score for species-level abundance showed that 15 species were found to change during bacterial vaginosis (Figure 2B), 11 which increased (*G. vaginalis,A. vaginae, Snethia amnii, Aerococcus christensenii, Prevotella amnii, Gemella asaccharolytica, Facklamia languida, Porphyromonas asaccharolytica, Porphyromonas somerae, Mycoplasma hominis*, and "*Urinacoccus timonensis*" [ID 411805 - BioProject - NCBI]) and four which decreased (*Staphylococcus haemolyticus, Escherichia coli, Lactobacillus crispatus*, and *Bacillus simplex*). It is notable that all the diminished taxa were aerotolerant species while all the augmented taxa were strictly or facultative anaerobes.

Microbial culturomics of healthy vaginal samples enabled the isolation of 261 bacteria distributed into seven phyla with predomination of *Firmicutes* (139), followed by 65 *Actinobacteria*, 40 *Bacteroidetes*, 14 *Proteobacteria*, and one species from each of the following phyla: *Epsilonbacteraeota*, *Fusobacteria,* and *Synergistetes*. Isolated bacteria were classified into 108 genera including *Lactobacillus* (15), *Staphylococcus* (13), *Anaerococcus* (11), *Peptoniphilus* (11), *Streptococcus* (11), *Actinomyces* (10), *Bacteroides* (10), and *Prevotella* (10). On the other hand, species isolated in women with bacterial vaginosis were classified into seven phyla: 133 *Firmicutes*, 59 *Actinobacteria*, 31 *Bacteroidetes*, nine *Proteobacteria*, two *Fusobacteria*, two *Synergistetes*, and one *Epsilonbacteraeota,* for a total of 94 genera. The major genera were *Streptococcus* (13)*, Peptoniphilus* (12), *Anaerococcus* (11), *Staphylococcus* (11)*, Clostridium* (10)*,* and *Prevotella* (10)*.* Bacteria cultured from the microbiota of women with bacterial vaginosis belonged mostly to the *Firmicutes* phylum (p=0.007, unpaired t-test) with a dominance of *Clostridium* and *Peptoniphilus* spp ($p \leq 0.02$, Mann Whitney test) (Table 3). Thus, completing the results from metagenomics, in addition to a decrease of lactobacilli and *Proteobacteria,* women with bacterial vaginosis also showed an increase of *Clostridium* and *Peptoniphilus* spp*.*

Variation in vaginal microbiota composition according to the area studied

To investigate whether there is an association between bacterial communities and ethnicity, the vaginal microbiota of Caucasian French women was compared to those of Senegalese women (Figure 3). According to the metagenomics results, rural Senegalese women exhibited a richer microbiota (p<0.05, two groups parametric ANOVA for Chao-1, and ACE) than that of Caucasian women, but there was no significant difference in diversity (p>0.05 for Shannon and Simpson indices) (Table 4). Strikingly, OTUs belonging to *Bacillus*, *Escherichia*, *Staphylococcus,* and *Corynebacterium* were abundant in healthy Caucasian women while rural Senegalese healthy women harboured diverse anaerobic species such as *Prevotella*, *Ureaplasma,* and *Peptoniphilus* (Figure 4). Culturomics results revealed that the vaginal microbiota was significantly richer and more diverse in Caucasian women. The number of isolated species in women with bacterial vaginosis (63.29±11.22 versus 30.6±11.48, p<0.0001) and healthy vaginas (43±10.33 versus 24.80±12.48, p=0.002) increased considerably in Caucasian women, with many anaerobic bacteria (Table 5).

Discussion

Recently, OMICS strategies have provided an overview of the composition of bacterial communities, their function, and their interaction with host cells. Despite being revolutionary, this does not replace knowledge gained through the isolation of bacterial microorganisms. Knowledge about the composition and diversity of the human vaginal microbiota in health and bacterial vaginosis conditions may be useful for a better understanding of this dysbiosis and for preventing or controlling it. Our study of 15 vaginal flora samples with bacterial vaginosis and 35 normal flora samples, aimed at exhaustively mapping the vaginal microbiota and understanding the putative link between the condition of bacterial vaginosis and microbial composition and diversity. Will this in mind, we simultaneously performed a strategy that has already been applied to vaginal flora, that of metagenomics [41, 42], and coupled it with a new one, culturomics [22]. One of the major findings is that the composition of vaginal microbiota changes considerably during bacterial vaginosis with a significant increase in bacterial diversity. Indeed, some studies have previously shown that bacterial vaginosis was obviously associated with more rich and diverse vaginal bacterial community [1, 43, 44], and the vaginal microbiota profile belongs to CST IVC and CST IVD [25], as corroborated in our study.

Of the nine phyla found in the vaginal samples using both culturomics and metagenomics, *Firmicutes* was by far the major phylum detected in both microbiotas (normal and bacterial vaginosis). Our results revealed a significant decrease in *Lactobacillaceae* and *Proteobacteria* species while bacterial taxa such as *G. vaginalis*, *A. vaginae*, *Aeroccocus christensenii, Prevotella, Peptoniphilus, Clostridium, Snethia amnii*, *Mycoplasma hominis, Porphyromonas, Facklamia languida*, and *Gemella asaccharolytica* increased. It is not surprising that the presence of bacterial vaginosis is marked by a depletion of protective *Lactobacillus* species and aerotolerant bacteria [17, 45–47], causing a transition in the vaginal microbiota from a state of eubiosis to that of dysbiosis [48]. Moreover, most of the non-*Lactobacillus* species found to be highly prevalent in patients with bacterial vaginosis have been already reported to be associated with bacterial vaginosis [1, 43, 49–52]. The first detection of *Facklamia languida* extends the number of BV-associated bacteria reported in the literature up to now [49–53]. Interestingly, *Facklamia* species have recently been associated with the etiology of numerous clinical infections [54]. The microbial population detected in bacterial vaginosis flora was greatly similar to bacterial communities found in human faeces, with the presence of bacteria belonging to the genera *Prevotella, Peptoniphilus*, and *Clostridium* [55]. Our data provided insight into some scientists interrogations [56, 57], with the hypothesis that the presence of gastrointestinal flora associated with bacterial vaginosis may be due to their inoculation during certain sexual practices. Thus, bacterial vaginosis is a dysbiosis that may result from the introduction into the vagina of bacteria from the anus, causing a change in pH which leads to a loss of vaginal equilibrium. Lactobacilli are then depleted, and the vaginal ecosystem becomes favourable to the proliferation of many resident anaerobic species such as *A. vaginae* and *G. vaginalis*.

Using metagenomics, we noticed that the vaginal microbiota of Senegalese women was richer than that of the Caucasian French women, with an abundance of anaerobic species. These results are in line with previous reports showing different bacterial diversity between women of Caucasian origin and those of African origin in the USA [24, 58, 59]. The culturomics results on the same samples revealed the opposite, but this difference between the data from metagenomics and culturomics can be explained by the lag time between the sample collection time and their culture. The French vaginal samples were immediately inoculated upon collection, thus enabling the isolation of a wide range of bacteria which were mainly anaerobic, while those from rural Senegalese women were stored at -80 °C for a few months before culturing. These data highlight that storage and transport are one drawback of culture strategies, with the loss and death of bacterial species especially anaerobic ones. By testing the viability of vaginal microorganisms in two and three commercial transport media, respectively at different temperatures and time points, Stoner *et al*., [60] and DeMarco *et al*., [61] noted that the microorganisms that grew depended upon the transport media used and also the temperature and time that had elapsed before analysis. They indicated that to prevent proliferation during transport and to maintain anaerobic vaginal bacteria, culture must be take place within 24 hours of being stored at 4 °C. Despite the limits of culture, it is important to underline that seven new species were obtained from the 10 frozen Senegalese samples (Table 2). Overall, it would be better to associate inoculation of the sample collected from the patient's 'bedside' with its preincubation in a culture bottle in order to isolate extremely sensitive bacteria.

Several studies have attempted to characterise the vaginal microbiota using different strategies including culture and molecular techniques [50, 62–64]. With metagenomic investigations, the number of phylotypes (previously uncultivated or new undiscovered) in the vagina microbiota was higher in women with bacterial vaginosis than in normal flora and was similar to previous molecular studies of vaginal microbiota which detected numerous uncultivated BV-associated bacteria such as BVAB1, BVAB2, *Megasphaera* sp type 1 and 2, *Dialister* sp type 1 to 3, and *Eggerthella* sp type 1 [50, 51, 64, 65]. Despite the huge contribution that metagenomics has made to studying microbiota diversity, some limitations remain to be addressed. These include the differences between the regions targeted in

different studies as well as the high throughput analysis tool and approaches requiring a certain level of expertise, which limits their use among the scientific community [66–68].

Conclusions

Our study is the first attempt to characterise the vaginal microbiota using the strategy of culturomics. Our findings revealed the complementary of metagenomics and culturomics approaches and made it possible to detect a total of 554 bacteria (with 360 identified by metagenomics and 340 isolated by culturomics, and only 146 common species detected using both techniques). Culturomics extended the repertoire of vaginal flora with the isolation of 142 bacterial species already detected in humans but never in the vaginal flora. Overall, 27 new bacterial species including ("*Peptoniphilus vaginalis"*, *"Megasphaera vaginalis"* and *"Atopobium massiliense"*) that were closely related to *Peptoniphilus* sp. DNF00840, *Megasphaera* sp. BV3C16-1 and *Atopobium* sp. S4-5 respectively, corresponded to BVABs and had previously been detected using only molecular tools. In contrast to classical culture methods [63], culturomics revealed its aptitude for bacterial isolation by almost doubling the number of cultivated species in the human gut [69, 70].

In summary, during an imbalance of the microbiota, it is important to know the impact of sampling and exploration techniques on the microbial community. In spite of its limitations, this study is the first to investigate the vaginal microbiota of women with and without bacterial vaginosis, in France and Senegal, and also the first to use culturomics. The metagenomics strategy targeting the 16S rRNA gene coupled with the culturomics strategy highlights the richness and diversity of the vaginal microbiota. Although culturomics do not cover all taxa in this microbial econiche, it has been successfully applied to isolate several sets of bacteria including three keys members of bacterial vaginosis flora: *Peptoniphilus* sp. DNF00840, *Megasphaera* sp. BV3C16-1, and *Atopobium* sp. S4-5, which were only previously detected by molecular tools. In the future, it would be interesting to continue with culturomics, increasing the number of vaginal samples analysed and diversifying the geographical study areas, as well as using new media mimicking the vaginal environment. Obtaining isolates will enable the *in vitro* exploration of the competition between bacteria from vaginal microbiota but will also serve as the raw material for developing a treatment by bacteriotherapy by proposing a cocktail of key bacteria to prevent or treat bacterial vaginosis.

Materials And Methods

Study design

This study focused on investing the vaginal microbiota of healthy women and those suffering from bacterial vaginosis. The project included 34 women living in two geographical areas: 24 from France (Public University Hospitals, Marseille) and 10 from rural Senegal (villages of Dielmo and Ndiop, Sine-Saloum area). Only non-pregnant, HIV-negative, 20-to 50-year-old pre-menopausal women who had received no antibiotic treatment in the two months preceding the study were eligible to participate.

The ethics committees of the Institut Fédératif de Recherche IFR48 (Marseille, France) and that of the Senegalese CNERS, in accordance with the SEN protocol 16/04, approved this study under agreement numbers 09-022 and 00039, respectively. All participants were volunteers and gave their informed written and signed consent.

Sample collection and study process

After an explanation, women collected their own vaginal discharges [71, 72] using Sigma Transwab (Medical Wire, Corsham, United Kingdom). A fresh culture was made immediately, within minutes of collection for swabs collected in France. Those sampled in rural Senegal were stored and transported to the laboratory in Dakar, the capital, in a portable freezer at -20°C. As soon as they arrived, they were stored at -80°C until they were sent to Marseille on dry ice. Once in Marseille, they were stored at -80°C until further analysis. In addition, we also collected 16 additional vaginal specimens from five of the French women at their follow-up visits (5, 5, 3, 2, and 1 respectively). Of the 50 specimens included in the study, all were analysed using metagenomics targeting thee 16S rRNA gene, but only 34 were analysed using microbial culturomics. The diagnosis of bacterial vaginosis was assessed by molecular quantification of the microorganisms *Atopobium vaginae* and *Gardnerellavaginalis*, as previously described, [73] in parallel to Nugent score [74].

High-throughput culture-based technique: culturomics

Culture conditions and isolation of bacteria

Vaginal bacterial communities were isolated using culturomics [22, 75]. Firstly, the samples were vortexed in 3 mL of Transwab, and 100 µL of the resulting broth was diluted in 900 µL of Dulbecco's phosphate-buffered saline (DPBS). Ten-fold cascade dilutions were performed and aliquots of 50 µL of each dilution were directly seeded onto five solid culture media: anaerobe basal agar (Oxoid, Dardilly, France), Colistin Nalidixic Acid agar, Columbia sheep blood agar, Schaedler agar enriched with sheep blood and vitamin K1, and Trypticase soy agar with horse blood (all four, BD Diagnostics, Le Pont-de-Claix, France). The Petri dishes were incubated at 37°C under anaerobic atmosphere for four to seven days. Then, briefly, 2 mL of the vaginal fluid was pre-incubated in both anaerobic and aerobic liquid culture medium (BD Diagnostics), supplemented with sterile blood (3 mL) and filtered rumen (4 mL), both from sheep. At different pre-incubation periods (1, 3, 7, 10, 15, 21, and 30 days) at 37°C, 100 µL of the broth was sampled using the format plating described below. The anaerobic bottle mixture was inoculated on the five media cited above then incubated for seven days under the same conditions, whereas aerobic broth was plated on Chocolate agar PolyViteX (BD Diagnostics) and incubated aerobically for three days at 37°C. Isolated bacteria were purified and then identified using MALDI–TOF mass spectrometry with a Microflex spectrometer (Bruker, Leipzig, Germany) [76, 77].

Rapid bacterial identification using MALDI-TOF mass spectrometry

Each purified colony was spotted in duplicate on a 96 MALDI-TOF target plate and covered with 2 µL of matrix solution, as previously reported [76, 77]. Bacterial identification was performed using the Microflex spectrometer which compares the obtained protein spectra with those present in the library (Bruker and URMS databases). Isolates with an unambiguous score ≥ 2.0 were considered as having been correctly identified at species level. For unidentified bacteria using MALDI-TOF mass spectrometry (score ≤ 2.0), 16S rRNA gene sequencing was performed to determine its taxonomic position [78].

Bacterial identification based on 16S rRNA gene sequencing

Bacterial DNA was extracted using the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was subjected to an amplification using primers FD1 and RP2 (Eurogentec, Angers, France) targeting a conserved bacteria region and an annealing temperature of 52°C. The amplified product was verified by electrophoresis gel migration prior to its purification and re-amplification using the BigDye Terminator sequencing kit (Qiagen, Courtaboeuf, France) with a system of eight primers (357F, 358R 536F, 536R, 800F, 800R, 1050 F, and 1050R). The amplicons were purified and sequenced using an ABI PRISM 3130-XL capillary sequencer (Applied Biosystems, Bedford, MA, USA). The obtained sequences were corrected and compared to those available in the GenBank database. Sequences with a nucleotide similarity percentage below 98.7% and 95% were considered as new species or genus, respectively [79, 80].

High-throughput molecular method: metagenomics

Extraction and metagenomics sequencing

DNA from vaginal samples was extracted using a mechanical treatment performed with acid washed powder glass beads (Sigma, Lyon, France) and 0.5 mm glass beads cell disruption media (Scientific Industries, Bohemia, NY, USA) using a FastPrep BIO 101 instrument (Qbiogene, Strasbourg, France) at maximum speed (6.5 m/sec) for 90 seconds. The specimens were then treated using two kinds of lyses methods: one method with classic lysis and a protease step following by purification on NucleoSpin Tissue kit (Macherey Nagel, Hoerdt, France), and the other using a deglycosylation step and purification on the EZ1 Advanced XL device (Qiagen) [41]. Samples were first amplified on these two extractions, pooled and barcoded, then sequenced for 16S rRNA sequencing on MiSeq technology (Illumina, San Diego, CA, USA) using the paired-end strategy, constructed according to the 16S metagenomic sequencing library preparation (Illumina). For each protocol extraction, metagenomic DNA was sequenced for the 16S rRNA gene's "V3-V4" regions as previously described [81]. All reads from these two methods were grouped and clustered with a threshold of 98% identity to obtain operational taxonomic units (OTUs). The paired reads were filtered according to the read qualities.

Taxonomic assignments of OTUs.

The paired-end sequences were assembled into longer sequences by Pandaseq [82]. The resulting fastq files of longer sequences ≥ 400 nts were then demultiplexed in the QIIME2 pipeline (Quantitative Insights Into Microbial Ecology) version 2018.2 [83]. The sequence quality was controlled and feature/OTU table was constructed in DADA2 [84] of QIIME2. The OTUs/Feature sequences were blasted [85] against the reference database of SSURef of Silva [86] and taxonomy was assigned by applying majority voting [81, 87], considering species level ≥ 98% identity and 100% coverage. The unassigned OTUs were then blasted against the IHU (Institut

Hospitalo-Universitaire) database containing all species isolated by culturomics. OTUs that remained unassigned to any species were regarded as “unclassified”.

Data and statistical analyses

For metagenomics data, α-diversity (ACE, Chao-1, Shannon, and Simpson indices) was calculated using MicrobiomeAnalyst [88]. Based on their differential abundance, microbial markers were determined using the Ward clustering method based on Euclidean distance [88]. The bacterial abundance profile among our two groups (healthy women and women with bacterial vaginosis) were identified using linear discriminant analysis (LDA) effect size (LEfSe) methods [89]. The statistically different features were then computed as a cladogram using GraPhlAn [90]. β-diversity (comparison of the number of taxa unique for each ecosystem) was also calculated for culture data. It constituted the ratio of the unique/totality of the microbiota of every group. Depending on the Gaussian distribution, *t*-test or Mann Whitney test was used to compare quantitative data, and exact Fischer or Chi-squared tests for proportions. Statistical analyses were performed using GraphPad Prism version 5.03 and values were presented as mean and standard deviation.

Declarations

Ethics approval and consent to participate

All participants gave their informed written and signed contentment. The study was approved by the ethics committees of the Institut Fédératif de Recherche IFR48 (Marseille, France) and

the Senegalese CNERS under agreement numbers 09-022 and 00039, respectively.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare. Funding sources had no role in the design and conduct of the study; the collection, management, analysis, and interpretation of the data; and the preparation, review, or approval of the manuscript.

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Authors’ contributions

KD, JCL, DR, and FF conceived and designed the experiments. KD, AL, NSF, ND, FB, CS, and FF contributed to the materials and analysis tools. KD and NSF perform the culturomics. KD, DB, AL, JCL, and FF analysed and interpreted the data. KD and FF wrote the paper. All authors read and approved the final manuscript.

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Tables

Table 1
Socio-demographic characteristic and vaginal microbiota profile of 34 women.

	Bacterial vaginosis (n = 12)	Normal flora (n = 22)	*p*-value
Age	29.39 ± 10.80	25.82 ± 8.82	NS
Ethnicity			NS
Caucasian	7 (58.3%)	17 (77.3%)	
Rural Senegalese	5 (41.7%)	5 (22.7%)	
Bacterial load			
Bacterial load $\log_{10}$ of 16S rRNA gene/sample	4.8 ± 0.4	4.9 ± 0.3	NS
Richness and diversity index			
Reads	99,568 ± 74,868	112,928 ± 66,466	NS
ACE[1]	34.8 ± 1.7	28.7 ± 1.5	NS
Chao-1	29.5 ± 16.5	22.5 ± 15.5	0.05
Shannon[2]	1.9 ± 0.7	1.3 ± 0.8	0.02
Simpson	0.3 ± 0.2	0.5 ± 0.3	0.02
Evenness[3]	0.14 ± 0.05	0.09 ± 0.05	0.009
Taxa exclusively found using both techniques			
	Bifidobacterium scardovii	*Acidaminococcus intestini*	
	Facklamia ignava	*Acinetobacter baumannii*	
		Alistipes putredinis	
		Bacteroides cellulosilyticus	
		Bacteroides fragilis	
		Bacteroides salyersiae	
		Enterococcus pallens	
		Lactobacillus mucosae	
		Macrococcus caseolyticus	
		Morganella morganii	
		Phascolarctobacterium faecium	
		Pseudoramibacter alactolyticus	
		Streptococcus australis	
		Streptococcus urinalis	
		Trueperella bernardiae	

[1]Richness (ACE and Chao-1) and [2]diversity (Shannon and Simpson) indices were evaluated using the MicrobiomeAnalyst pipeline. [3]Shannon evenness was calculated using the formula: E = H/ln(S) with H = Shannon index and S = total number of sequences in that cohort. *P*-value is shown only for a significant relationship. NS: not significant (p > 0.05).

Table 2
Growth conditions, taxonomic and, the source of putative new species isolated in the human vagina.

Phylogenetic affiliation	Species name	Strain	First culture condition	Sample origin	Clinical status	16S accession number	Relatedness to known species
Actinobacteria							
Actinomycetaceae	*Varibaculum vaginae*	Marseille-P5644	Direct plating, 5% sheep blood trypticase soy agar, anaerobe, 37 °C	Dielmo, Senegal	Normal flora	LS999997	98% *Varibaculum cambriense*
	Arcanobacterium ihumii	Marseille-P5647	Incubation in a blood culture for 20 days, 5% sheep blood CNA agar, anaerobe, 37 °C	Dielmo, Senegal	Normal flora	LT993248	96.64% *Arcanobacterium phocae* LT629804
Atopobiaceae	*Atopobium massiliense*	Marseille-P4126	Direct plating, 5% sheep blood CNA and trypticase soy agar, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT986001	98.19% *Atopobium vaginae* AF325325
	*Olegusella massiliensis**	KHD7	Incubation in a blood culture for 10 days, 5% sheep blood CNA agar, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LN998058	93.5% *Olsenella uli* NR_074414
Coriobacteriaceae	*Collinsella vaginalis*	Marseille-P2666	Incubation in a blood culture for 15 days, 5% sheep blood CNA agar, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT598547	96.08% *Collinsella intestinalis* NR_113165
Corynebacteriaceae	*Corynebacterium feminarum*	Marseille-P4858	Direct plating, 5% sheep blood trypticase soy agar, anaerobe, 37 °C	Marseille, France	Normal flora	LS999987	98.2% *Corynebacterium similans*

*New genus.

Phylogenetic affiliation	Species name	Strain	First culture condition	Sample origin	Clinical status	16S accession number	Relatedness to known species
	Corynebacterium fournierii	Marseille-P2948	Incubation in a blood culture for 30 days, Chocolate agar PVX, aerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT576414	98.7% *Corynebacterium ureicelerivorans* NR_042558
Eggerthellaceae	*Vaginimassilia timonensis**	Marseille-P4307	Direct plating, 5% sheep blood CNA agar, anaerobe, 37 °C	Dielmo, Senegal	Bacterial vaginosis	LT996087	93.4% *Gordonibacter urolithinfaciens* LT900217
Intrasporangiaceae	*Janibacter massiliensis*	Marseille-P4121	Incubation in a blood culture for 10 days, Schaedler agar with 5% sheep blood and vitamin K, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT969384	98.01% *Janibacter melonis* JN644568
Propionibacteriaceae	*Cutibacterieum timonense*	Marseille-P5998	Incubation in a blood culture for 15 days, 5% sheep blood trypticase soy agar, anaerobe, 37 °C	Dielmo, Senegal	Normal flora	LT996136	97.80% *Cutibacterium acnes* CP023676
	Tessaracoccus timonensis	Marseille-P5995	Direct plating, 5% sheep blood CNA agar, anaerobe, 37 °C	Dielmo, Senegal	Bacterial vaginosis	LT996088	97.30% *Tessaracoccus oleiagri* GU111567
	*Vaginimicrobium propionicum**	Marseille-P3275	Incubation in a blood culture for 10 days, 5% sheep blood CNA agar, Schaedler and Trypticase soy agar, anaerobe, 37 °C	Marseille, France	Normal flora	LT598595	92.92% *Propionimicrobium lymphophilum* LT223675
Bacteroidetes							

*New genus.

Phylogenetic affiliation	Species name	Strain	First culture condition	Sample origin	Clinical status	16S accession number	Relatedness to known species
Flavobacteriaceae	*Vaginella massiliensis**	Marseille-P2517	Incubation in a blood culture for 7 days, Chocolate agar PVX, aerobe, 37 °C	Marseille, France	Normal flora	LT223570	93.03% *Weeksella virosa* NR_074495
Prevotellaceae	*Prevotella lascolaii*	KhD1	Incubation in a blood culture for 24 hours, Schaedler agar with 5% sheep blood and vitamin K, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LN998055	90% *Prevotella loescheii* FJ717335
Firmicutes							
Aerococcaceae	*Vaginisenegalia massiliensis**	Marseille-P5643	Direct plating, 5% sheep blood CNA agar, anaerobe, 37 °C	Dielmo, Senegal	Bacterial vaginosis	LT971014	93.77% *Facklamia hominis* NR_026393
Lactobacillaceae	*Lactobacillus raoultii*	Marseille-P4006	Incubation in a blood culture for 3 days, 5% sheep blood agar, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT854294	98.1% *Lactobacillus farraginis* AB690214
Peptoniphilaceae							
	Anaerococcus mediterraneensis	Marseille-P2765	Incubation in a blood culture for 21 days, Schaedler agar with 5% sheep blood and vitamin K, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT598544	97.2% *Anaerococcus lactolyticus* NR_113565
	Anaerococcus genitaliorum	Marseille-P3625	Incubation in a blood culture for 10 days, 5% sheep blood CNA agar and Schaedler, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT900366	93.37% *Anaerococcus tetradius* GQ422749

*New genus.

Phylogenetic affiliation	Species name	Strain	First culture condition	Sample origin	Clinical status	16S accession number	Relatedness to known species
	Anaerococcus mediannikovii	Marseille-P3915	Incubation in a blood culture for 10 days, 5% sheep blood agar, anaerobe, 37 °C	Marseille, France	Normal flora	LT966066	96,73% *Anaerococcus lactolyticus* NR_113565
	Ezakiella massiliensis	Marseille-P2951	Incubation in a blood culture for 21 days, 5% sheep blood CNA agar, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT576398	98.5% *Ezakiella peruensis* KJ469554
	Helcococcus massiliensis	Marseille-P4590	Incubation in a blood culture for 15 days, 5% sheep blood agar, anaerobe, 37 °C	Dielmo, Senegal	Bacterial vaginosis	LT934442	95.5% *Helcococcus seattlensis* NR_118641
	*Khoudiadiopia massiliensis**	Marseille-P2746	Incubation in a blood culture for 21 days, Schaedler agar with 5% sheep blood and vitamin K, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT598561	89.28% *Murdochiella asaccharolytica* EU483153
	Murdochiella vaginalis	Marseille-P2341	Incubation in a blood culture for 15 days, Schaedler agar with 5% sheep blood and vitamin K, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT576397	97.3% *Murdochiella asaccharolytica* NR_116331
	Peptoniphilus pacaensis	Kh-D5	Incubation in a blood culture for 15 days, 5% sheep blood CNA and trypticase soy agar, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LN998072	97.2% *Peptoniphilus coxii* NR_117556

*New genus.

Phylogenetic affiliation	Species name	Strain	First culture condition	Sample origin	Clinical status	16S accession number	Relatedness to known species
	Peptoniphilus raoultii	KHD4	Incubation in a blood culture for 24 hours, 5% sheep blood CNA and trypticase soy agar, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LN998068	96% *Peptoniphilus lacrimalis* NR_041938
	Peptoniphilus vaginalis	KhD-2	Incubation in a blood culture for 24 hours, 5% sheep blood CNA and trypticase soy agar, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LN907856	98.2% *Peptoniphilus harei* NR_026358
Veillonellaceae							
	Megasphaera vaginalis	Marseille-P4857	Direct plating, 5% sheep blood CNA, anaerobe, 37 °C	Marseille, France	Bacterial vaginosis	LT960586	95.23% *Megasphaera micronuciformis* GU470904
*New genus.							

Table 3
Comparison of the microbial culturomics results of vaginal bacterial diversity between patients with bacterial vaginosis and healthy women.

Vaginal flora	Bacterial vaginosis (n = 12)	Normal flora (n = 22)	p-value
Global Diversity			
Phyla	4.7 ± 1.6	4.2 ± 1.2	0.45[a]
Genera	30 ± 10	25 ± 9	0.26[b]
Species	50 ± 20	39 ± 13	0.06[b]
New species	1.5 ± 2	0.4 ± 0.6	0.03[a]
Diversity by Phylum			
Firmicutes	28 ± 9.8	20 ± 6.2	0.007[b]
Actinobacteria	13 ± 5.2	11 ± 4.8	0.25[b]
Bacteroidetes	5.7 ± 5.1	4.8 ± 4.2	0.70[a]
Proteobacteria	1.7 ± 1.3	1.5 ± 1.4	0.64[b]
Fusobacteria	0.2 ± 0.4	0.2 ± 0.4	0.70[a]
Synergistetes	0.2 ± 0.6	0.04 ± 0.2	0.66[a]
Epsilonbacteraeota	0.5 ± 0.5	0.4 ± 0.5	0.63[a]
Diversity by Genus			
Peptoniphilus	4.5 ± 2.8	2.2 ± 1.9	0.009[a]
Anaerococcus	2.5 ± 1.7	1.8 ± 1.8	0.20[b]
Prevotella	2.0 ± 1.9	1.1 ± 1.5	0.11[a]
Clostridium	1.3 ± 1.1	0.5 ± 0.7	0.02[a]
Lactobacillus	1.6 ± 1.6	2.2 ± 1.4	0.28[a]
Bifidobacterium	1.2 ± 1.2	0.5 ± 0.8	0.07[b]
Atopobium	1.2 ± 1.2	0.9 ± 0.8	0.48[b]
Gardnerella	0.5 ± 0.5	0.4 ± 0.5	0.81[b]

[a] Mann Whitney test. [b] unpaired *t*-test.

Table 4

Metagenomics comparison of vaginal microbiota between French and rural Senegalese women, with and without bacterial vaginosis (BV).

	Reads	ACE	Chao-1	Shannon	Simpson
BV-France (n = 7)	88,001	17 ± 1.30	22.9 ± 9.10	1.9 ± 0.20	0.28 ± 0.07
BV-Senegal (n = 5)	115,762	25.4 ± 1.60	39.6 ± 20.60	2.02 ± 0.40	0.29 ± 0.10
Healthy-France (n = 17)	106,792	48 ± 1.98	18.06 ± 3.20	1.2 ± 0.20	0.46 ± 0.28
Healthy-Senegal (n = 5)	133,792	37.4 ± 1.60	35.08 ± 5.70	1.4 ± 0.40	0.47 ± 0.29
p-value (ANOVA)	NS	0.003	0.007	NS	NS
NS: not significant ($p > 0.05$).					

Table 5

Comparison of vaginal species isolated from French and rural Senegalese women, with and without bacterial vaginosis (BV).

Global diversity	French women	Senegalese women	*p*-value
Bacterial vaginosis (n = 12)	**7**	**5**	
Phyla	5.3 ± 0.9	3.8 ± 1.9	0.09[a]
Total species	63.3 ± 11.2	30.6 ± 11.5	0.0006[b]
Strictly anaerobic species	38 ± 11.1	15.2 ± 11.9	0.005[b]
Aerotolerant species	25.4 ± 5.7	15.6 ± 2.7	0.005[b]
Healthy flora (n = 22)	**17**	**5**	
Phyla	4.8 ± 0.8	2.4 ± 0.5	0.0009[b]
Species	43 ± 10.3	24.8 ± 12.5	0.002[b]
Strictly anaerobic species	24.2 ± 9.5	8.8 ± 9	0.004[b]
Aerotolerant species	18.8 ± 3.8	15.4 ± 4.4	0.10[b]
[a] Mann Whitney test. [b] unpaired *t*-test.			

Figures

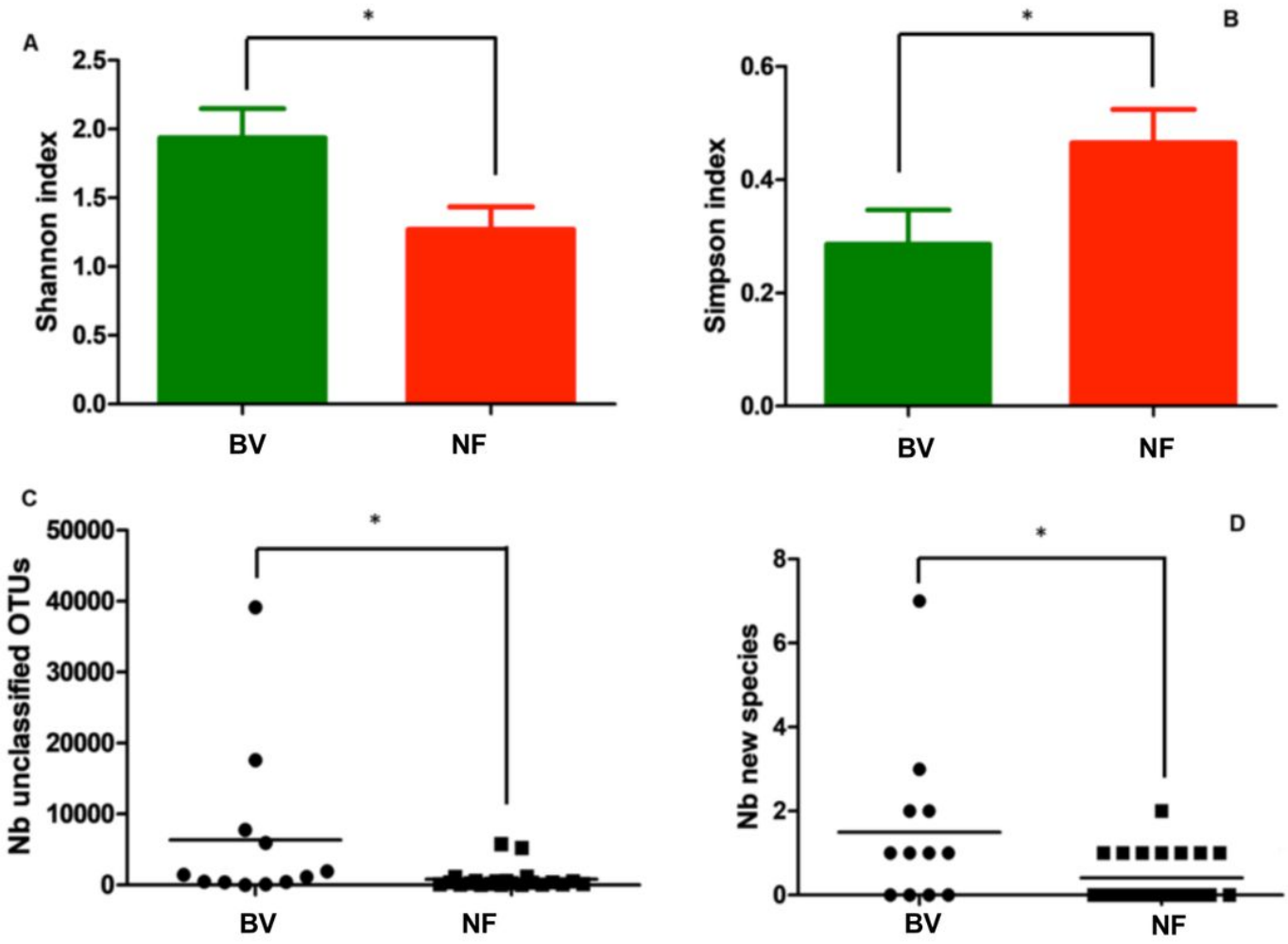

Figure 1

Comparison of vaginal microbiota diversity between normal flora (NF) and bacterial vaginosis (BV). Species diversity estimated by Shannon (A) and Simpson indices (B). The hitherto unknown diversity detected using metagenomics (C) and culturomics (D) approaches. Women with bacterial vaginosis showed a significantly increased (known as well as previously unknown) diversity. *p < 0.05.

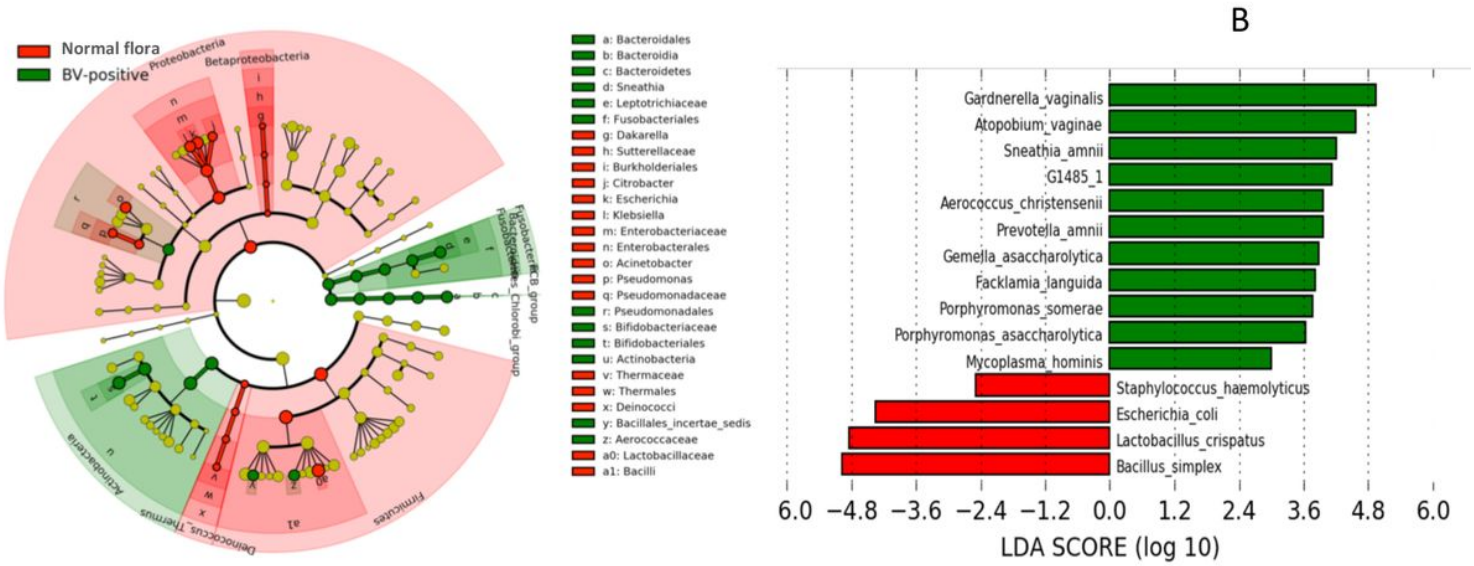

Figure 2

Identification of biomarker taxa between bacterial vaginosis (BV)-positive and normal vaginal microbiota. A. Cladogram representation of taxa with different relative abundance in accordance with BV status. B. LDA score (linear discriminant analysis) showing abundant species as biomarkers in BV-positive and normal vaginal microbiota.

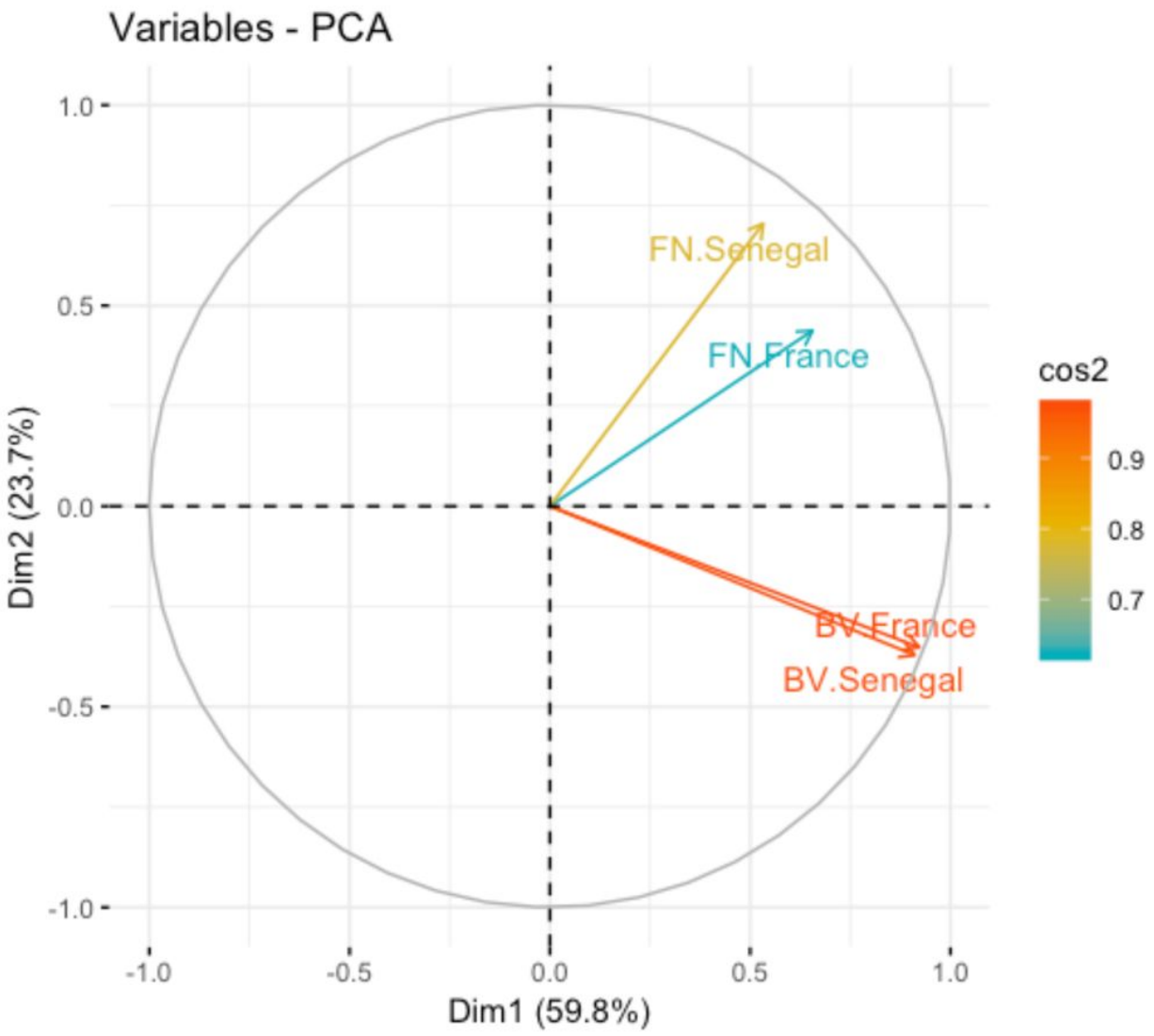

Figure 3

Principal Components Analysis (PCA) graph showing the correlation of the vaginal microbiota status (Bacterial vaginosis [BV] or normal flora [NF]) and the geographical repartition (France or Senegal) for 34 vaginal samples.

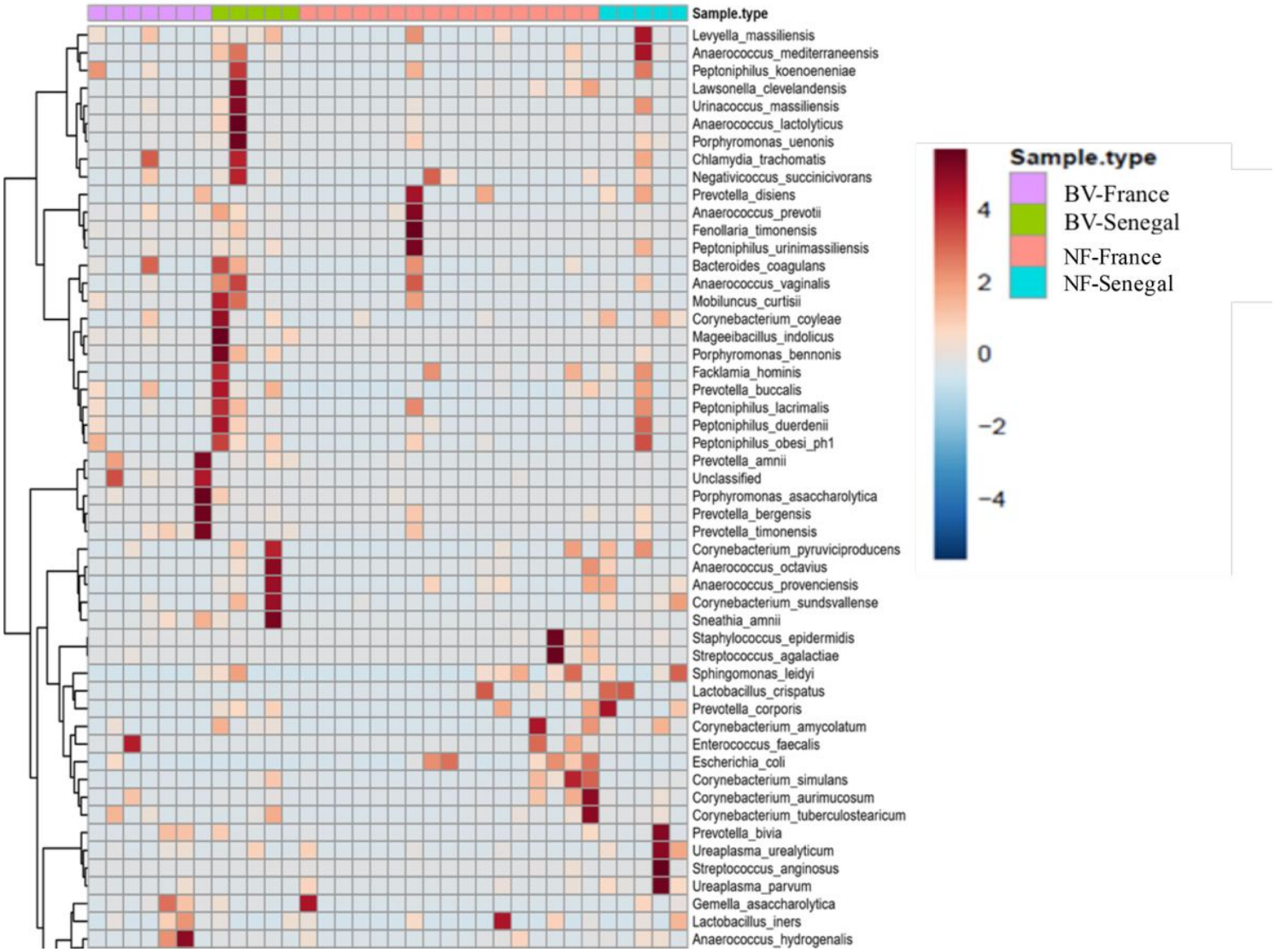

Figure 4

Heatmap by using the Ward clustering method based on the Euclidean distance.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS2.xlsx
- TableS1.xlsx