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Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis

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Abstract

Purpose. In the present study we sought to find novel bacterial and metabolic hallmarks for bacterial vaginosis (BV).

Methods. We studied the vaginal microbiome and metabolome of vaginal fluids from BV-affected patients (n = 43) and healthy controls (n = 37) by means of an integrated approach based on quantitative PCR (qPCR) and proton Nuclear Magnetic Resonance (^{1}H-NMR). The correlations between the clinical condition and vaginal bacterial communities were investigated by principal component analysis (PCA). To define the metabolomics signatures of BV, 100 discriminant analysis by projection on latent structure (PLS-DA) models were calculated.

Results. Bacterial signatures distinguishing the health condition and BV were identified by qPCR. *L. crispatus* strongly featured the healthy vagina while increased concentrations of *Prevotella*, *Atopobium* and *Mycoplasma hominis* specifically marked the infection. ^{1}H-NMR analysis has led to the identification and quantification of 17 previously unreported molecules. BV was associated with changes in the concentration of metabolites belonging to the families of amines, organic acids, short chain fatty acids, amino acids, nitrogenous bases and monosaccharides. In particular, maltose, kynurenine and NAD+ primarily characterized the healthy status while nicotinate, malonate and acetate were the best metabolic hallmarks of BV.

Conclusions. This study helps to better understand the role of the vaginal microbiota and metabolome in the development of BV infection. We propose a molecular approach for diagnosis of BV based on quantitative detection in vaginal fluids of *Atopobium*, *Prevotella* and *Mycoplasma hominis*, and nicotinate, malonate and acetate by combining qPCR and ^{1}H-NMR.

Keywords: vaginal microbiome; vaginal metabolome; bacterial vaginosis; lactobacilli; qPCR; ^{1}H-NMR

Introduction

The microbiota of the human vagina can significantly impact the health of women, their fetuses and newborn infants [1]. The vaginal microbiota of healthy reproductive age women is dominated by *Lactobacillus* species. Five distinct vaginal bacterial biotypes, characterized by the dominance of *L. crispatus*, *L. gasseri*, *L. iners*, *L. jensenii*, or an increased proportion of other strictly anaerobic bacteria, were described [2, 3]. Lactobacilli play key protective roles through different mechanisms, such as production of various antibacterial compounds, co-aggregation, competitive exclusion and immunomodulation [4-6].

Bacterial vaginosis (BV) is a common polymicrobial disorder of the vaginal microbiota characterized by loss of lactobacilli and increasing numbers of anaerobes and gram-negative rods [7, 8]. BV is associated with adverse outcomes, such as ascending reproductive tract infections, enhanced acquisition of HIV and other sexually transmitted diseases, spontaneous abortion and preterm birth [9-12]. Both a defined etiology and optimal treatment strategies for BV have remained elusive.

BV is typically diagnosed using either the Nugent scoring method [13] that examines bacterial composition via a Gram smear or the Amsel criteria [14] that considers factors such as presence of discharge, amine production, presence of clue cells and a vaginal pH greater than 4.5.

The microbiology of BV has been better characterized through microbiome studies based on 16S rRNA gene-directed PCR assays [15]. Although these approaches are able to provide a comprehensive understanding of the bacterial community membership, they are not able to determine the changes occurring in the vaginal environment at a metabolic level. Metabolomics analyzes complex systems, using high-throughput analytical methods, such as NMR spectroscopy that allows robust and sensitive identification of metabolites produced by microbes and host cells. This tool allows researchers to determine the effects caused by perturbations on the host's metabolic profile by analyzing the presence

and quantity of thousands of metabolites simultaneously. Metabolites that are significantly affected by experimental variables can be identified by multivariate statistics and placed into the larger context of how the host was affected overall [16-19].

In the perspective to discern potential novel determinants of BV, we investigated the relationship between bacterial community composition and metabolic profiles of healthy and BV-associated vaginal ecosystems by means of quantitative PCR (qPCR) of bacterial 16S rRNA genes and proton Nuclear Magnetic Resonance (^{1}H-NMR)-based metabolomics.

Materials and Methods

Study participants and sample preparation

Subjects for this study were a cohort of 80 Belgian pre-menopausal, non-pregnant women, aged between 18 and 50 years (mean age: 37). The enrolled women belonged to two groups: BV-affected patients (n = 43) presenting positivity for at least three of four Amsel's criteria and a Nugent score > 3, and age-matched healthy subjects (n = 37) who had no signs of vaginal tract infection and had never had BV. All women tested negative to *Candida*, sexually transmitted infections and abnormal findings on cervical Pap smears. They signed an informed consent in accordance with the approval and guidelines of the Ethics committee of the Heilig Hart Hospital of Tienen, Belgium.

Standardized vaginal fluids were collected by flushing and re-aspirating 2 ml of saline through a 22-gauge needle in the left, central, and right upper vaginal vaults [20] and stored at -80°C until use. Vaginal samples were centrifuged at 9,500 × g for 15 min. The supernatants were used for metabolomic analysis, while the pellets were processed for DNA isolation by using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) [21, 22]. DNA amount was quantified using NanoDrop ND-

1000 (NanoDrop® Technologies, Wilmington, DE).

Quantitative PCR (qPCR)

qPCR was performed on DNA samples extracted from the vaginal fluids using a LightCycler instrument (Roche, Mannheim, Germany) and SYBR Green I as the reporter fluorophore. Genus- or species- specific primer sets targeted to 16S rRNA gene or 16S-23S rRNA spacer region were used to amplify bacteria belonging to *L. crispatus* [23], *L. iners* [24], *L. gasseri* [25], *L. jensenii* [24], *Gardnerella vaginalis* [26], *Atopobium* [27], *Prevotella* [28], *Veillonella* [29], *Mycoplasma hominis* [23] and *Mobiluncus* [30].

Amplifications were carried out in a final volume of 20 µl containing each primer at 0.5 µM, 4 µl of LightCycler-FastStart DNA Master SYBR green I (Roche) and 2 µl of template. The thermal cycling conditions were optimized in the present study (Table S1, supplemental material). DNAs extracted from *L. crispatus* DSM 20584, *L. gasseri* DSM 20243, *L. jensenii* DSM 20557, *L. iners* DSM 13335, *Gardnerella vaginalis* ATCC 14018, *Prevotella bivia* ATCC 29303, *Veillonella parvula* ATCC 10790, *Atopobium vaginae* ATCC BAA-55, *Mycoplasma hominis* DSM 19104, and *Mobiluncus curtisii* ATCC 43063 were used as standards for PCR quantification. The efficiency of qPCR was between 90% and 100%, and correlation coefficients for genomic DNA standards were > 0.99. The sensitivity of qPCR assays ranged from 10^{-4} to 10^{-1} ng of target DNA. Melting curve analysis was carried out to confirm that the PCR products from vaginal samples and standards had identical melting points.

DNA samples were amplified in triplicate for each primer set. Data were expressed as log ng of DNA of the target genus or species per µg of total DNA extracted from the vaginal sample.

^{1}H-NMR analysis

One ml of vaginal supernatant was added to 160 µl of a D_2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 6.25 mM and the pH was adjusted to 7.00 with the addition of HCl or NaOH (0.5 M). ^{1}H-NMR spectra were recorded at 298 K with an AVANCE spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz, equipped with an autosampler with 60 holders [17]. Each spectrum was acquired using 32K data points over a 7,211.54 Hz spectral width and adding 256 transients. A recycle delay of 5 s and a 90° pulse of 11.4 s were set up. Acquisition time (2.27 s) and recycle delay were adjusted to be 5 times longer than the longitudinal relaxation time of the protons under investigation, which was considered to be not longer than 1.4 s.

^{1}H-NMR spectra baseline was adjusted by means of the simultaneous peak detection [31] and baseline correction algorithm (SPDBC) implemented in the baseline R package [32]. Signals misalignments were compensated by i-Coshift algorithm [33]. Differences in water concentration were compensated by probabilistic quotient normalization procedure (PQN) [34]. The signals with an intensity of at least five times that of noise were assigned by comparing their chemical shift and multiplicity with the literature [17] and Chenomx software data bank (Chenomx Inc., Canada, ver 8.1). Their area was employed for molecular quantification.

Statistical analysis

Statistical analyses were performed using R computational language [35]. Similarities among the analyzed samples, as well as trends in the bacterial communities or metabolome profiles, were investigated by means of principal components analysis (PCA) applied to the mean centered data. Differences in bacterial amounts, metabolites concentrations and PCA data were analyzed using Wilcoxon's signed rank test and Anova based on Tukey contrast [36]. A P value < 0.05 was considered

statistically significant. In addition, to better define the metabolomics signatures of BV, 100 discriminant analysis by projection on latent structure (PLS-DA) models in their sparse version were calculated by means of mixOmics R package [37]. PLS-DA algorithm builds linear models with each molecule concentration in order to maximize the possibility to predict BV presence. In its sparse version, the most informative molecules are progressively added to the model until the best compromise between prediction ability and model simplicity is reached. The robustness of the models was expressed in terms of correct classification rate (CCR), that is the ratio between sum of true positives and negatives predictions and the total number of samples.

Results

Distribution of bacterial communities

A qualitative analysis of the microbiota composition in healthy and BV-affected women is represented in Fig. 1. The frequency of occurrence of *Lactobacillus* species and BV-related bacteria represented the first element to clearly distinguish the two vaginal ecosystems (Fig. 1a). Healthy women primarily harboured lactobacilli. Among these, *L. crispatus* was the most frequently detected bacterium (70.27%), followed by *L. gasseri* (45.94%), *L. iners* (24.32%) and *L. jensenii* (18.92%). The detection rate of *L. iners* was higher in BV-affected women (62.79%) than in healthy controls. The most frequently BV-related bacteria detected in healthy women were *Prevotella* (67.57%), *M. hominis* (62.16%) and *Atopobium* (54.05%). *G. vaginalis* was found in a small percentage (8.11%), while *Veillonella* and *Mobiluncus* were not detected. BV patients possessed a diverse array of bacteria, with the most frequent genera and species being *Atopobium* (100%), *G. vaginalis* (97.67%), *Prevotella* (97.67%) and *M. hominis* (83.72%). Despite *Veillonella* (41.86%) and *Mobiluncus* (23.26%) have been

found at a lower frequency than the other BV-related bacteria, their presence seemed to indicate unequivocally the existence of BV disorder. Differences in frequency of occurrence between healthy and BV-affected women were significant for all bacterial groups ($P < 0.05$), except for *L. jensenii*.

The relative abundance of the main *Lactobacillus* species associated with human vaginal mucosa has been explored as an additional criterion for distinguishing between health and BV (Fig. 1b). The majority of women was characterized by the presence of a single *Lactobacillus* species. For women who harboured more than one *Lactobacillus* species, the predominant species was identified based on the bacterial amount calculated by qPCR. The vaginal microbiota of the totality of healthy women was colonized by at least one *Lactobacillus* species. *L. crispatus* (59%) highly prevailed over other lactobacilli, followed by *L. iners* (22%), *L. gasseri* (16%), and *L. jensenii* (3%). Unlike health condition, the vaginal microbiota of a fair number of BV patients was not colonized by any of the four main *Lactobacillus* species (N: 33%) and *L. iners* was the predominant species (63%). The microbiota of a low percentage of BV-affected women was dominated by *L. gasseri* (5%), while *L. jensenii* and *L. crispatus* were never predominant.

Quantification of *Lactobacillus* species and BV-related bacteria

The qPCR results are summarized in box blots representing concentrations of specific DNA of *Lactobacillus* species and BV-related bacteria in vaginal samples of healthy controls and BV patients (Fig. 2).

Regarding lactobacilli, only *L. crispatus* showed a significant difference between healthy and BV women: the amount of this species was higher in healthy group (7.75 log ng/µg) than in BV group (6.47 log ng/µg) ($P < 0.0001$). As already reported [38], *L. iners* had the highest median concentration of any assayed species/genus in samples from both healthy (8.49 log ng/µg) and BV (8.58 log ng/µg)

191 women.

192 With respect to BV-related bacteria, the median concentrations of *Atopobium* (H: 0.13 log ng/µg; BV:
193 1.96 log ng/µg; $P < 0.0001$), *Prevotella* (H: -1.24 log ng/µg; BV: 1.20 log ng/µg; $P < 0.0004$) and *M.*
194 *hominis* (H: -1.22 log ng/µg; BV: 0.67 log ng/µg; $P < 0.0413$) were significantly higher in BV patients
195 compared to healthy controls. The amount of *G. vaginalis* did not vary significantly in the two groups
196 of women. *Veillonella* (-1.07 log ng/µg) and *Mobiluncus* (1 log ng/µg) were identified only in BV cases
197 at low concentrations.

198

199 Metabolic profiles

200

201 We sought a metabolic description of BV compared to health condition. 55 signals ascribable to single
202 molecules were identified (Table S2, supplemental material). 44 of these molecules were identified and
203 quantified, while other 6 were identified, but not reliably quantified, due to signals superimpositions or
204 artifacts introduced by signal alignment procedure. Concerning citrate, BV condition was not
205 associated to a variation in concentration, but to a shift in signals in the spectrum, a well-known
206 behavior caused by oscillations in divalent cations concentrations [39]. We identified and quantified 17
207 previously unreported small molecules, including the organic acids malonate and isovalerate, the
208 amino acids histidine, taurine and aspartate, the nitrogenous bases NAD+ and inosine.
209 Among the identified and quantified molecules, 32 significantly varied in concentration between
210 healthy and BV women ($P < 0.05$). These metabolites belong to the families of amines, organic acids,
211 short chain fatty acids (SCFAs), amino acids, nitrogenous bases and monosaccharides (Table 1). BV
212 was associated with a general increase of amines, organic acids and various SCFAs. BV was also
213 characterized by a general decrease of protein amino acids, with the exception of proline. Within the
214 other categories of metabolites no uniform trend of variation was observed.

215

216 Microbiome-metabolome correlation

217

218 The correlations between the clinical condition and vaginal bacterial communities have been studied by
219 means of a PCA built on the qPCR data related to *Lactobacillus* species and BV-related bacteria (Fig.
220 S1, supplemental material). The first two PCs accounted for the 32.3% of the whole variance of the
221 investigated samples.
222 Changes in bacterial populations were highlighted by plotting the medians of the two groups of women
223 (H and BV). H and BV groups were significantly different ($P < 0.05$), showing that most of the
224 variability was due to the onset of BV condition. BV samples were more widely distributed in the
225 multidimensional space compared to healthy samples, indicating a greater inter-individual variability of
226 microbiome and metabolome, as previously hypothesized [17, 40]. The vaginal microbiota of control
227 women was dominated by *L. crispatus*, *L. gasseri* and *L. jensenii* with the former most strongly
228 characterizing the state of health. *L. iners* was shifted toward the disease condition, suggesting its
229 correlation with the development of BV. The bacterial groups that primarily featured women with BV
230 were *Prevotella* and *Atopobium*, followed by *M. hominis*. Conversely, *Mobiluncus* and *Veillonella*,
231 which were identified in small quantities only in presence of BV, played a minor role as biomarkers of
232 the disease. The results of PCA show that concentration is a relevant factor in determining a distinctive
233 feature of BV in terms of diagnostic applications. *G. vaginalis* did not appear as one of the species most
234 closely associated with BV.
235 The implications of BV on vaginal metabolome were investigated by building 100 sPLS-DA models on
236 the metabolites that significantly varied in BV women compared to healthy controls (Fig. 3). At least in
237 90 times the variable reduction algorithm selected maltose, kynurenine, nicotinate, malonate, acetate
238 and NAD^+ as the most important metabolites in discriminating vaginal health from BV. In particular,

maltose, kynurenine and NAD+ were distinctive of healthy condition while nicotinate, malonate, and acetate appeared to be specific hallmarks of BV. These molecules were also characterized by median variable influence on projections (VIP) coefficients higher than 1, feature that was shared with sarcosine and phenylalanine. PLS-DA model built on these 6 molecules only gave a percentage of 0.11 ± 0.42 (mean ± standard deviation) of misclassified samples pertaining to BV-affected women, and no misclassified test samples pertaining to healthy women. This optimal performance was obtained in all cases, including women dominated by *L. iners*. This means that, despite these women presented a BV-like microbiome, the conditions regulating the concentration of maltose, kynurenine, nicotinate, malonate, acetate and NAD^+ represent a common trait of BV onset and could be reliably used as biomarkers and to better understand the biochemical bases of this disease.

Correlation between *Lactobacillus* species, BV-related bacteria and metabolites

In order to go into deeper detail about the health-promoting potential of *L. crispatus*, *L. iners*, *L. gasseri* and *L. jensenii*, the correlations between the dominance of each *Lactobacillus* species and the vaginal microbiome and metabolome were analyzed by PCA (Fig. S2, supplemental material). In the biplot describing the correlations between the dominant *Lactobacillus* species and BV-related bacteria, PC1 and PC2 accounted for the 47.4% of the whole variance of the investigated samples (Fig. S2a). Medians of the four healthy groups were superimposed while the median of BVn was significantly different from the medians of BVi and BVg ($P < 0.05$), showing that the variability of BV samples was not only associated to the development of BV but also to the presence of lactobacilli. The more different microbiota from health condition was found in BVn, followed by BVi. An intermediate position in the two-dimensional space was occupied by BVg.

The six molecules best discriminating healthy condition from BV allowed to obtain a visual

correlations between the predominance of each *Lactobacillus* species and metabolome, as depicted in Fig. S2b. PC1 and PC2 accounted for the 67.9% of the whole variance of the investigated samples. The distribution in the PC plane of the samples from BV-affected women closely reproduced the trends observed in Fig. S2a, with BVg samples appearing as most similar to the healthy samples, BVn samples most different, and *L. iners* prevalence giving rise on average to an intermediate condition.

Discussion

In the present study we sought to identify correlations between the vaginal colonization of certain bacterial populations and metabolic profiles, in order to find novel hallmarks of BV.

Our results support previous studies that BV is associated with dramatic compositional changes in the vaginal microbiota, i.e. depletion of lactobacilli in conjunction with colonization of many diverse bacteria, mainly strict anaerobes [7, 20, 39, 41, 42]. Healthy women primarily harbored lactobacilli: *L. crispatus* was the most frequent species, detected at significantly higher concentration with respect to BV patients. The frequency of occurrence of *L. iners* was higher in BV-affected women, confirming the hypothesis that this species is common and abundant in vaginal communities characterized by high concentrations of non-lactobacilli [22, 42]. Other studies of perturbed vaginal microflora suggest that *L. iners* might be a transitional species, colonizing after disturbances to the vaginal environment [43].

Compared to healthy controls, BV patients had in their vaginal microbiota higher prevalence of all the considered BV-related bacteria and significantly higher abundances of *Atopobium*, *Prevotella* and *M. hominis*. Because *Atopobium*, *Prevotella* and *M. hominis* were also frequently represented in healthy vagina (> 50%), high levels rather than the mere presence of these bacteria can be considered hallmarks of BV disorder. *G. vaginalis*, which has long been considered distinctive of BV [44], in our study did not seem to be particularly useful for diagnostic purposes. In fact, neither the presence nor an increased

concentration of this species were uniquely associated with the disease. The results of multivariate statistical analysis reiterated the role of *L. crispatus* in strongly featuring the healthy vagina and the functions of *Prevotella*, *Atopobium* and *M. hominis* as specific microbio-markers of BV disorder. Conversely, *Mobiluncus* and *Veillonella*, even though highly specific for BV, seemed to play a minor role in marking the disease, probably because the majority of BV-affected women did not harbor these bacteria. It remains to be elucidated whether *Mobiluncus/Veillonella* positive BV expresses a subtype of BV with specific features and pathogenicity. Notably, PCA highlighted the relevance of bacterial concentration in determining a distinctive feature for proper diagnosis of BV.

The molecular data descriptive of the vaginal microbiome have been integrated with the ^{1}H-NMR data descriptive of the metabolic activities of the host-bacteria meta-organisms, in order to obtain a comprehensive picture of the evolution of the vaginal ecosystem associated with the infection. This led to the identification and quantification of 17 previously unreported small molecules, including the organic acids malonate and isovalerate, the amino acids histidine, taurine and aspartate, the nitrogenous bases NAD^{+} and inosine.

BV was associated with changes in several metabolites belonging to the families of amines, organic acids, short chain fatty acids, amino acids, nitrogenous bases and monosaccharides. Most of these molecules are known to be modulated by the balance between lactobacilli and BV-related bacteria, such as biogenic amines, SCFAs and organic acids [17, 45-49]. In particular, we have observed in BV cases a general increase of amines, including tyramine, trimethylamine and cadaverine, which are considered the main responsible for the fishy odor of the vaginal discharge [8, 19]. Various SCFAs were significantly higher in BV-positive women, which is not surprising given that BV is characterized by the outgrowth of mostly anaerobic bacteria. It has been suggested that SCFAs may be involved in recruitment and activation of the innate immune cells in the female genital tract [47]. This is in agreement with the recent finding that BV is associated with an increase of proteins involved in the

311 innate immune response [50]. Organic acids were detected at higher concentrations in the vaginal fluids
312 of BV-positive women, as previously noted especially for acetate and succinate, typical metabolites
313 produced by *Prevotella* and *Mobiluncus* spp. [45]. In the present work, malonate also assumes a
314 particular emphasis being together acetate the organic acids that mostly differ BV from healthy state.
315 BV was also characterized by a decrease of certain protein amino acids, probably due to their
316 decarboxylation to biogenic amines. The high availability of maltose in healthy vagina can be regarded
317 as the principal cause of the overgrowth of lactobacilli. It could be attributed to an efficient synthesis of
318 glycogen and/or secretion of α-amylase by healthy vaginal epithelium. In fact, the human α-amylase is
319 primarily responsible for the digestion of vaginal glycogen, making available glycogen-breakdown
320 products, including maltose [51]. On the other hand, we found an increase of glucose concentration in
321 BV-affected patients. This finding is in agreement with previous studies reporting the association of
322 dietary indices, i.e. glycemic load, with BV acquisition and persistence [52]. The high level of glucose
323 could favor the glycolysis which involves the consumption of NAD^+.
324 In conclusion, our work may help to better understand the role of the vaginal microbiota and
325 metabolome in BV infection. The identification of bacterial and metabolic markers described here is a
326 prerequisite for the design of new diagnostic kits, which, being based on molecular parameters, are
327 likely to be endowed with greater sensitivity and reproducibility compared to the methods of diagnosis
328 currently used in clinical practice (Amsel and Nugent). Specifically, we propose a molecular tool for
329 diagnosis of BV based on quantitative detection in vaginal fluids of *Atopobium*, *Prevotella* and
330 *Mycoplasma hominis*, and malonate, acetate, and nicotinate by combining qPCR and ^{1}H-NMR. The
331 combination of these selected bacterial/ metabolic hallmarks could provide the best diagnostic test in
332 terms of specificity, allowing to correctly diagnose cases of infection reducing false positive rate.
333 Regarding sensitivity, such a combination maintains the peculiarities of qPCR and ^{1}H-NMR
334 techniques, that are able to detect bacterial DNA and metabolites at concentrations below the ng and

mmol/l, respectively.

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Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Figure captions

Fig. 1 Molecular analysis of the vaginal microbiota composition in healthy (H) and BV-affected (BV) women. (a) Frequency of occurrence of *L. crispatus*, *L. iners*, *L. gasseri*, *L. jensenii*, *Atopobium*, *G. vaginalis*, *Prevotella*, *Veillonella*, *Mobiluncus* and *M. hominis*, calculated as the percentage of women hosting each bacterial group. **, $P < 0.01$; *, $P < 0.05$. (b) Relative abundance of *L. crispatus*, *L. iners*, *L. gasseri* and *L. jensenii*, calculated as percentage of women who hosted each species as dominant. (the predominant species was identified based on the bacterial amount calculated by qPCR). N indicate the percentage of women that is not colonized by any of the four analysed species

Fig. 2 Quantification of *L. crispatus*, *L. iners*, *L. gasseri*, *L. jensenii*, *Atopobium*, *G. vaginalis*, *Prevotella*, *Veillonella*, *Mobiluncus* and *M. hominis* in healthy (H) and BV-affected (BV) women. qPCR data are expressed as log ng of DNA of the target genus or species per µg of total DNA extracted from the vaginal sample. The box for each bacterial group represents the interquartile range (25^{th} to 75^{th} percentile), and the line within this box is the median value. Bottom and top bars indicate the 10^{th} and 90^{th} percentiles, respectively. Outlier values are indicated (black circles). Only positive samples for each bacterial group analysed were considered. **, $P < 0.01$; *, $P < 0.05$

Fig. 3 Frequency of inclusion of the 32 molecules of Table 1 calculated by the sPLS-DA predictive model. The median variable influence on projections (VIP) coefficients are indicated in parentheses

Supplemental material

Fig. S1 Biplot of a PCA performed on the autoscaled qPCR data. Median values of the samples groups corresponding to healthy and BV-affected women are indicated as H and BV. Empty circles and filled squares indicate samples from healthy and BV women, respectively. Expl. Var, explained variance

Fig. S2 Biplot of a PCA performed on the autoscaled qPCR data related to BV-associated bacteria (a) and metabolites selected by sPLS-DA model. (b) Median values of the samples groups corresponding to healthy women dominated by *L. crispatus*, *L. iners*, *L. gasseri*, *L. jensenii* and BV-affected women dominated by *L. iners*, *L. gasseri* or none of the four considered species are indicated as Hc, Hi, Hg, Hj, BVi, BVg and BVn, respectively. Empty circles and filled squares indicate samples from healthy and BV women, respectively. Expl. Var, explained variance

530 **Table 1** Concentration of metabolites which significantly varied ($P < 0.05$) in BV-affected patients
531 (BV) compared to healthy control women (H)

Molecule	Chemical shift (ppm)	H	BV	Variations in BV
Amines				
Tyramine	7.228	$1.52x10^{-02} \pm 1.29x10^{-02}$	$9.21x10^{-02} \pm 1.02x10^{-01}$	↑
Ethanolamine	3.151	$6.04x10^{-02} \pm 1.85x10^{-02}$	$1.18x10^{-01} \pm 5.29x10^{-02}$	↑
TMA	2.894	$6.50x10^{-04} \pm 7.13x10^{-04}$	$2.13x10^{-02} \pm 1.99x10^{-02}$	↑
Methylamine	2.61	$9.08x10^{-04} \pm 8.91x10^{-04}$	$1.97x10^{-02} \pm 2.12x10^{-02}$	↑
Cadaverine	1.722	$1.64x10^{-01} \pm 6.66x10^{-02}$	$3.80x10^{-01} \pm 3.15x10^{-01}$	↑
Organic acids				
Formate	8.459	$3.35x10^{-02} \pm 7.96x10^{-02}$	$4.50x10^{-01} \pm 6.88x10^{-01}$	↑
Malonate	3.134	$1.74x10^{-02} \pm 7.29x10^{-03}$	$4.98x10^{-02} \pm 2.56x10^{-02}$	↑
Succinate	2.409	$1.91x10^{-01} \pm 1.63x10^{-01}$	$9.70x10^{-01} \pm 1.12x10^{+00}$	↑
Pyruvate	2.380	$1.22x10^{-01} \pm 1.48x10^{-01}$	$3.38x10^{-01} \pm 3.91x10^{-01}$	↑
Acetate	1.916	$7.24x10^{-01} \pm 5.33x10^{-01}$	$4.73x10^{+00} \pm 3.12x10^{+00}$	↑
SCFAs				
Propionate	2.200	$1.56x10^{-02} \pm 9.42x10^{-03}$	$4.21x10^{-01} \pm 7.61x10^{-01}$	↑
Butyrate	2.150	$5.03x10^{-02} \pm 1.95x10^{-02}$	$1.62x10^{-01} \pm 2.68x10^{-01}$	↑
2-Hydroxyisovalerate	0.837	$2.01x10^{-02} \pm 1.02x10^{-02}$	$3.87x10^{-02} \pm 2.56x10^{-02}$	↑
Amino acids				
Tryptophan	7.749	$1.25x10^{-02} \pm 5.95x10^{-03}$	$9.20x10^{-03} \pm 6.66x10^{-03}$	↓
Phenylalanine	7.391	$9.46x10^{-02} \pm 3.15x10^{-02}$	$6.80x10^{-02} \pm 3.43x10^{-02}$	↓
Tyrosine	7.191	$6.77x10^{-02} \pm 2.53x10^{-02}$	$4.54x10^{-02} \pm 3.51x10^{-02}$	↓
Glutamate	2.354	$3.07x10^{-01} \pm 1.28x10^{-01}$	$1.77x10^{-01} \pm 8.86x10^{-02}$	↓

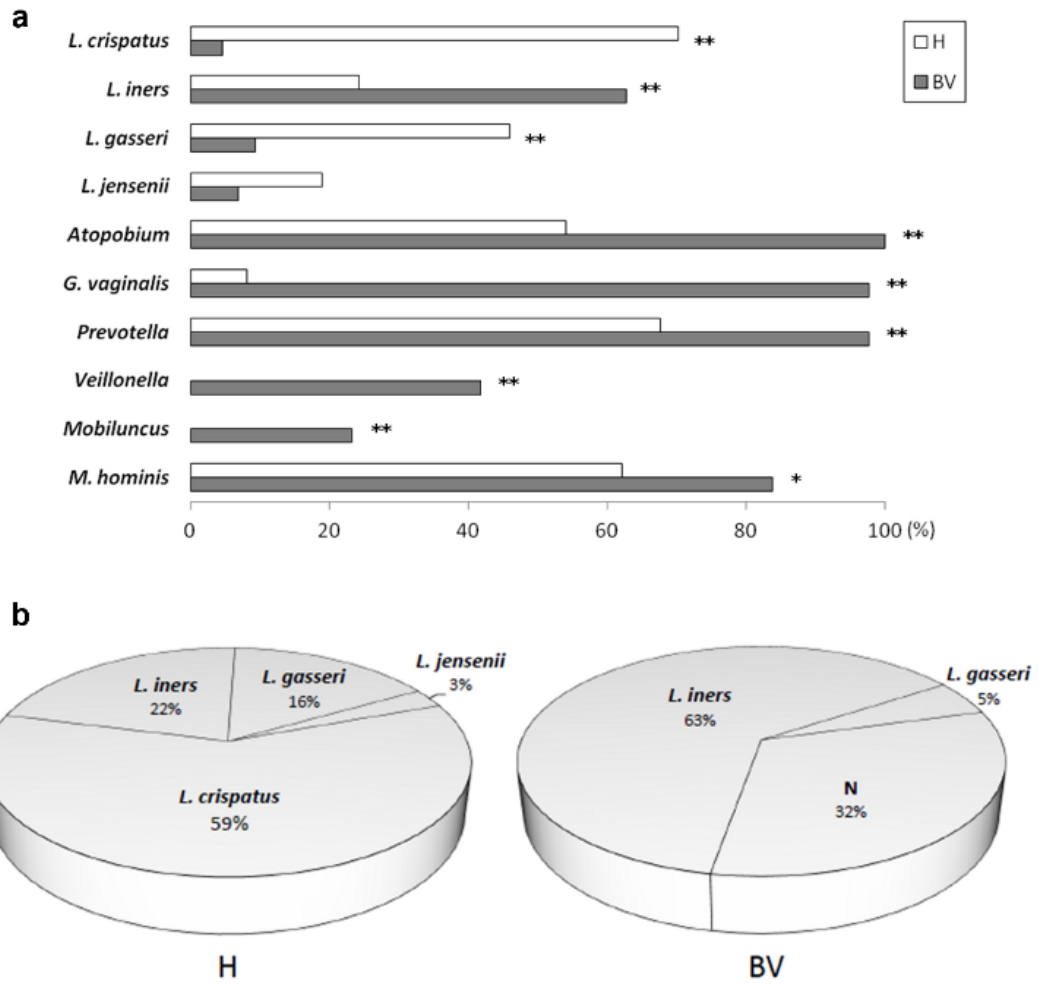
Proline	2.017	$2.95x10^{-02} \pm 1.19x10^{-02}$	$4.70x10^{-02} \pm 2.88x10^{-02}$	↑
Isoleucine	1.012	$8.27x10^{-02} \pm 3.54x10^{-02}$	$6.91x10^{-02} \pm 8.39x10^{-02}$	↓
Leucine	0.962	$4.65x10^{-01} \pm 1.95x10^{-01}$	$3.66x10^{-01} \pm 3.43x10^{-01}$	↓
Nitrogenous bases				
NAD+	9.341	$2.22x10^{-03} \pm 1.58x10^{-03}$	$1.83x10^{-04} \pm 1.28x^{-03}$	↓
Nicotinate	8.943	$1.63x10^{-03} \pm 1.30x10^{-03}$	$4.77x10^{-03} \pm 1.92x10^{-03}$	↑
Uracil	7.543	$1.32x10^{-02} \pm 6.01x10^{-03}$	$3.12x10^{-02} \pm 3.06x10^{-02}$	↑
Inosine	6.098	$3.61x10^{-03} \pm 7.46x10^{-03}$	$8.12x10^{-04} \pm 1.48x10^{-03}$	↓
Sugars				
Glucose	3.537	$8.27x10^{-02} \pm 6.48x10^{-02}$	$1.67x10^{-01} \pm 1.28x10^{-01}$	↑
Maltose	3.298	$1.17x10^{+00} \pm 6.51x10^{-01}$	$6.27x10^{-01} \pm 5.10x10^{-01}$	↓
Others				
Urocanate	6.410	$6.15x10^{-04} \pm 1.45x10^{-03}$	$3.84x10^{-03} \pm 4.07x10^{-03}$	↑
Kynurenine	6.812	$5.72x10^{-02} \pm 2.33x10^{-02}$	$1.94x10^{-02} \pm 1.84x10^{-02}$	↓
sn-Glycero-3-phosphocholine	3.229	$2.45x10^{-02} \pm 1.11x10^{-02}$	$1.35x10^{-02} \pm 1.72x10^{-02}$	↓
Sarcosine	2.757	$2.99x10^{-02} \pm 1.78x10^{-02}$	$1.17x10^{-02} \pm 6.89x10^{-03}$	↓
2-Aminoadipate	2.241	$8.66x10^{-02} \pm 6.09x10^{-02}$	$2.58x10^{-01} \pm 1.55x10^{-01}$	↑
3-Methyl-2-oxovalerate	1.109	$3.34x10^{-03} \pm 3.39x10^{-03}$	$1.06x10^{-02} \pm 1.17x10^{-02}$	↑

532 Values are expressed as means of mmol/l ± standard deviation.

533 TMA, Trimethylamine; SCFAs, short chain fatty acids.

534

535 Figure 1
536



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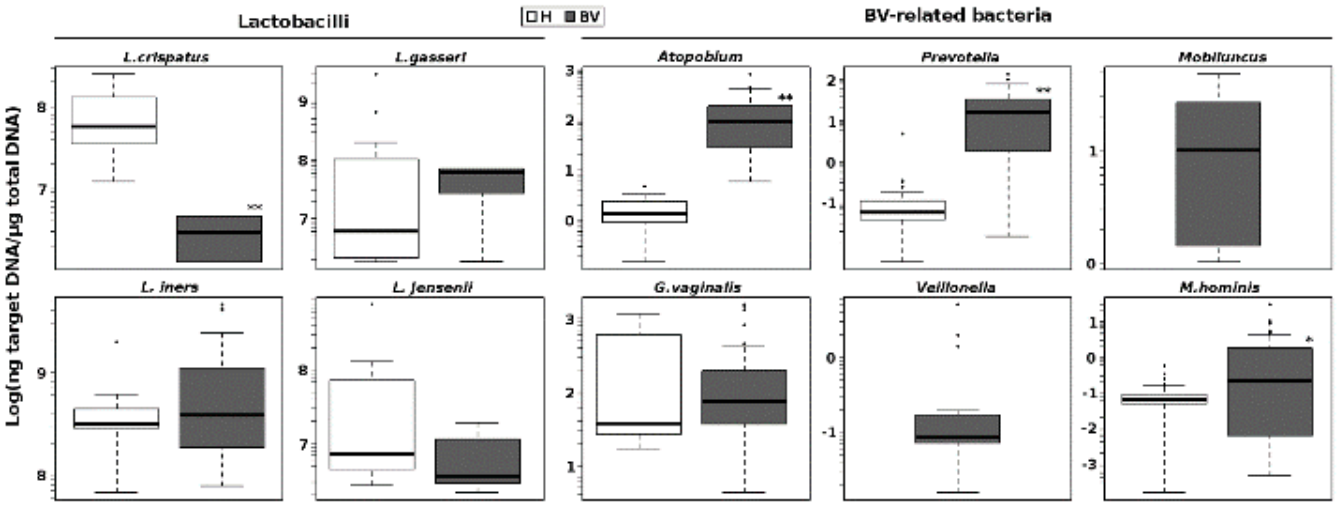
540 Figure 2



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543 Figure 3

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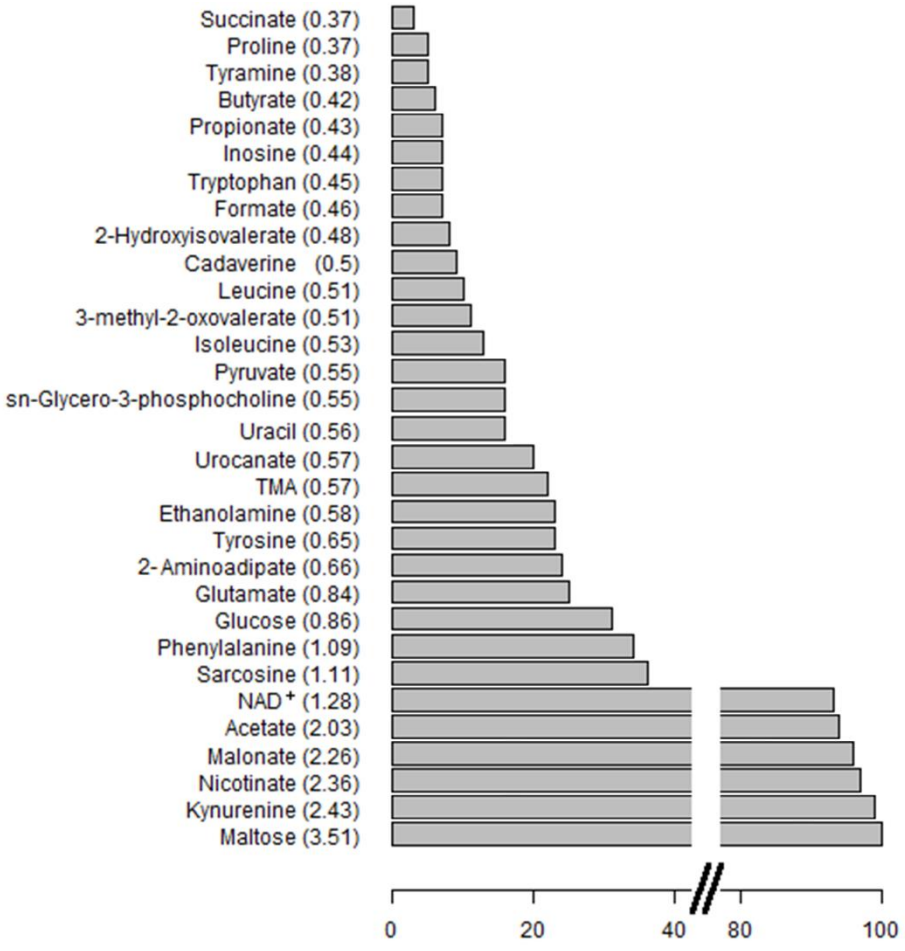

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