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Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis

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**Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis**

**by Beatrice Vitali, Federica Cruciani, Gianfranco Picone, Carola Parolin, Gilbert Donders, Luca Laghi**

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1 **Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis**

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24 **Abstract**

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

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

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

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

45 **Keywords:** vaginal microbiome; vaginal metabolome; bacterial vaginosis; lactobacilli; qPCR;  $^1\text{H-NMR}$

46

## 47 **Introduction**

48

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

56 Bacterial vaginosis (BV) is a common polymicrobial disorder of the vaginal microbiota characterized  
57 by loss of lactobacilli and increasing numbers of anaerobes and gram-negative rods [7, 8]. BV is  
58 associated with adverse outcomes, such as ascending reproductive tract infections, enhanced  
59 acquisition of HIV and other sexually transmitted diseases, spontaneous abortion and preterm birth [9-  
60 12]. Both a defined etiology and optimal treatment strategies for BV have remained elusive.

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

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

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

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

78

## 79 **Materials and Methods**

80

### 81 Study participants and sample preparation

82

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

90 Standardized vaginal fluids were collected by flushing and re-aspirating 2 ml of saline through a 22-  
91 gauge needle in the left, central, and right upper vaginal vaults [20] and stored at -80°C until use.

92 Vaginal samples were centrifuged at  $9,500 \times g$  for 15 min. The supernatants were used for  
93 metabolomic analysis, while the pellets were processed for DNA isolation by using a DNeasy blood  
94 and tissue kit (Qiagen, Hilden, Germany) [21, 22]. DNA amount was quantified using NanoDrop ND-

95 1000 (NanoDrop<sup>®</sup> Technologies, Wilmington, DE).

96

97 Quantitative PCR (qPCR)

98

99 qPCR was performed on DNA samples extracted from the vaginal fluids using a LightCycler  
100 instrument (Roche, Mannheim, Germany) and SYBR Green I as the reporter fluorophore. Genus- or  
101 species- specific primer sets targeted to 16S rRNA gene or 16S-23S rRNA spacer region were used to  
102 amplify bacteria belonging to *L. crispatus* [23], *L. iners* [24], *L. gasseri* [25], *L. jensenii* [24],  
103 *Gardnerella vaginalis* [26], *Atopobium* [27], *Prevotella* [28], *Veillonella* [29], *Mycoplasma hominis*  
104 [23] and *Mobiluncus* [30].  
105 Amplifications were carried out in a final volume of 20 µl containing each primer at 0.5 µM, 4 µl of  
106 LightCycler-FastStart DNA Master SYBR green I (Roche) and 2 µl of template. The thermal cycling  
107 conditions were optimized in the present study (Table S1, supplemental material). DNAs extracted  
108 from *L. crispatus* DSM 20584, *L. gasseri* DSM 20243, *L. jensenii* DSM 20557, *L. iners* DSM 13335,  
109 *Gardnerella vaginalis* ATCC 14018, *Prevotella bivia* ATCC 29303, *Veillonella parvula* ATCC 10790,  
110 *Atopobium vaginae* ATCC BAA-55, *Mycoplasma hominis* DSM 19104, and *Mobiluncus curtisii* ATCC  
111 43063 were used as standards for PCR quantification. The efficiency of qPCR was between 90% and  
112 100%, and correlation coefficients for genomic DNA standards were > 0.99. The sensitivity of qPCR  
113 assays ranged from 10<sup>-4</sup> to 10<sup>-1</sup> ng of target DNA. Melting curve analysis was carried out to confirm  
114 that the PCR products from vaginal samples and standards had identical melting points.  
115 DNA samples were amplified in triplicate for each primer set. Data were expressed as log ng of DNA  
116 of the target genus or species per µg of total DNA extracted from the vaginal sample.

117

118 <sup>1</sup>H-NMR analysis

119

120 One ml of vaginal supernatant was added to 160  $\mu$ l of a D<sub>2</sub>O solution of 3-(trimethylsilyl)-propionic-  
121 2,2,3,3-d<sub>4</sub> acid sodium salt (TSP) 6.25 mM and the pH was adjusted to 7.00 with the addition of HCl or  
122 NaOH (0.5 M). <sup>1</sup>H-NMR spectra were recorded at 298 K with an AVANCE spectrometer (Bruker,  
123 Milan, Italy) operating at a frequency of 600.13 MHz, equipped with an autosampler with 60 holders  
124 [17]. Each spectrum was acquired using 32K data points over a 7,211.54 Hz spectral width and adding  
125 256 transients. A recycle delay of 5 s and a 90° pulse of 11.4 s were set up. Acquisition time (2.27 s)  
126 and recycle delay were adjusted to be 5 times longer than the longitudinal relaxation time of the protons  
127 under investigation, which was considered to be not longer than 1.4 s.

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

135

136 Statistical analysis

137

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

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

151

## 152 **Results**

153

### 154 Distribution of bacterial communities

155

156 A qualitative analysis of the microbiota composition in healthy and BV-affected women is represented  
157 in Fig. 1. The frequency of occurrence of *Lactobacillus* species and BV-related bacteria represented the  
158 first element to clearly distinguish the two vaginal ecosystems (Fig. 1a). Healthy women primarily  
159 harboured lactobacilli. Among these, *L. crispatus* was the most frequently detected bacterium  
160 (70.27%), followed by *L. gasseri* (45.94%), *L. iners* (24.32%) and *L. jensenii* (18.92%). The detection  
161 rate of *L. iners* was higher in BV-affected women (62.79%) than in healthy controls. The most  
162 frequently BV-related bacteria detected in healthy women were *Prevotella* (67.57%), *M. hominis*  
163 (62.16%) and *Atopobium* (54.05%). *G. vaginalis* was found in a small percentage (8.11%), while  
164 *Veillonella* and *Mobiluncus* were not detected. BV patients possessed a diverse array of bacteria, with  
165 the most frequent genera and species being *Atopobium* (100%), *G. vaginalis* (97.67%), *Prevotella*  
166 (97.67%) and *M. hominis* (83.72%). Despite *Veillonella* (41.86%) and *Mobiluncus* (23.26%) have been

167 found at a lower frequency than the other BV-related bacteria, their presence seemed to indicate  
168 unequivocally the existence of BV disorder. Differences in frequency of occurrence between healthy  
169 and BV-affected women were significant for all bacterial groups ( $P < 0.05$ ), except for *L. jensenii*.  
170 The relative abundance of the main *Lactobacillus* species associated with human vaginal mucosa has  
171 been explored as an additional criterion for distinguishing between health and BV (Fig. 1b). The  
172 majority of women was characterized by the presence of a single *Lactobacillus* species. For women  
173 who harboured more than one *Lactobacillus* species, the predominant species was identified based on  
174 the bacterial amount calculated by qPCR. The vaginal microbiota of the totality of healthy women was  
175 colonized by at least one *Lactobacillus* species. *L. crispatus* (59%) highly prevailed over other  
176 lactobacilli, followed by *L. iners* (22%), *L. gasseri* (16%), and *L. jensenii* (3%). Unlike health  
177 condition, the vaginal microbiota of a fair number of BV patients was not colonized by any of the four  
178 main *Lactobacillus* species (N: 33%) and *L. iners* was the predominant species (63%). The microbiota  
179 of a low percentage of BV-affected women was dominated by *L. gasseri* (5%), while *L. jensenii* and *L.*  
180 *crispatus* were never predominant.

181

182 Quantification of *Lactobacillus* species and BV-related bacteria

183

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

187 Regarding lactobacilli, only *L. crispatus* showed a significant difference between healthy and BV  
188 women: the amount of this species was higher in healthy group (7.75 log ng/ $\mu$ g) than in BV group  
189 (6.47 log ng/ $\mu$ g) ( $P < 0.0001$ ). As already reported [38], *L. iners* had the highest median concentration  
190 of any assayed species/genus in samples from both healthy (8.49 log ng/ $\mu$ g) and BV (8.58 log ng/ $\mu$ g)

191 women.

192 With respect to BV-related bacteria, the median concentrations of *Atopobium* (H: 0.13 log ng/μg; BV: 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. hominis* (H: -1.22 log ng/μg; BV: 0.67 log ng/μg;  $P < 0.0413$ ) were significantly higher in BV patients compared to healthy controls. The amount of *G. vaginalis* did not vary significantly in the two groups of women. *Veillonella* (-1.07 log ng/μg) and *Mobiluncus* (1 log ng/μg) were identified only in BV cases 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 molecules were identified (Table S2, supplemental material). 44 of these molecules were identified and quantified, while other 6 were identified, but not reliably quantified, due to signals superimpositions or artifacts introduced by signal alignment procedure. Concerning citrate, BV condition was not associated to a variation in concentration, but to a shift in signals in the spectrum, a well-known behavior caused by oscillations in divalent cations concentrations [39]. We identified and quantified 17 previously unreported small molecules, including the organic acids malonate and isovalerate, the amino acids histidine, taurine and aspartate, the nitrogenous bases NAD<sup>+</sup> and inosine.

209 Among the identified and quantified molecules, 32 significantly varied in concentration between healthy and BV women ( $P < 0.05$ ). These metabolites belong to the families of amines, organic acids, short chain fatty acids (SCFAs), amino acids, nitrogenous bases and monosaccharides (Table 1). BV was associated with a general increase of amines, organic acids and various SCFAs. BV was also characterized by a general decrease of protein amino acids, with the exception of proline. Within the 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  $\text{NAD}^+$  as the most important metabolites in discriminating vaginal health from BV. In particular,

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

249

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

251

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

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

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

268

## 269 **Discussion**

270

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

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

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

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

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

301 BV was associated with changes in several metabolites belonging to the families of amines, organic  
302 acids, short chain fatty acids, amino acids, nitrogenous bases and monosaccharides. Most of these  
303 molecules are known to be modulated by the balance between lactobacilli and BV-related bacteria, such  
304 as biogenic amines, SCFAs and organic acids [17, 45-49]. In particular, we have observed in BV cases  
305 a general increase of amines, including tyramine, trimethylamine and cadaverine, which are considered  
306 the main responsible for the fishy odor of the vaginal discharge [8, 19]. Various SCFAs were  
307 significantly higher in BV-positive women, which is not surprising given that BV is characterized by  
308 the outgrowth of mostly anaerobic bacteria. It has been suggested that SCFAs may be involved in  
309 recruitment and activation of the innate immune cells in the female genital tract [47]. This is in  
310 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  $\alpha$ -amylase by healthy vaginal epithelium. In fact, the human  $\alpha$ -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  $\text{NAD}^+$ .

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  $^1\text{H-NMR}$ . 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  $^1\text{H-NMR}$   
334 techniques, that are able to detect bacterial DNA and metabolites at concentrations below the ng and

335 mmol/l, respectively.

336

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338 Research, Italy).

339

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

341

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

345

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493

494 **Figure captions**

495

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

503

504 **Fig. 2** Quantification of *L. crispatus*, *L. iners*, *L. gasseri*, *L. jensenii*, *Atopobium*, *G. vaginalis*,  
505 *Prevotella*, *Veillonella*, *Mobiluncus* and *M. hominis* in healthy (H) and BV-affected (BV) women.  
506 qPCR data are expressed as log ng of DNA of the target genus or species per  $\mu\text{g}$  of total DNA extracted  
507 from the vaginal sample. The box for each bacterial group represents the interquartile range (25<sup>th</sup> to 75<sup>th</sup>  
508 percentile), and the line within this box is the median value. Bottom and top bars indicate the 10<sup>th</sup> and  
509 90<sup>th</sup> percentiles, respectively. Outlier values are indicated (black circles). Only positive samples for  
510 each bacterial group analysed were considered. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$

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512

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

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518 **Supplemental material**

519

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

523

524 **Fig. S2** Biplot of a PCA performed on the autoscaled qPCR data related to BV-associated bacteria (a)  
525 and metabolites selected by sPLS-DA model. (b) Median values of the samples groups corresponding  
526 to healthy women dominated by *L. crispatus*, *L. iners*, *L. gasseri*, *L. jensenii* and BV-affected women  
527 dominated by *L. iners*, *L. gasseri* or none of the four considered species are indicated as Hc, Hi, Hg, Hj,  
528 BVi, BVg and BVn, respectively. Empty circles and filled squares indicate samples from healthy and  
529 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)

<b>Molecule</b>	<b>Chemical shift (ppm)</b>	<b>H</b>	<b>BV</b>	<b>Variations in BV</b>
<b>Amines</b>				
Tyramine	7.228	$1.52 \times 10^{-02} \pm 1.29 \times 10^{-02}$	$9.21 \times 10^{-02} \pm 1.02 \times 10^{-01}$	↑
Ethanolamine	3.151	$6.04 \times 10^{-02} \pm 1.85 \times 10^{-02}$	$1.18 \times 10^{-01} \pm 5.29 \times 10^{-02}$	↑
TMA	2.894	$6.50 \times 10^{-04} \pm 7.13 \times 10^{-04}$	$2.13 \times 10^{-02} \pm 1.99 \times 10^{-02}$	↑
Methylamine	2.61	$9.08 \times 10^{-04} \pm 8.91 \times 10^{-04}$	$1.97 \times 10^{-02} \pm 2.12 \times 10^{-02}$	↑
Cadaverine	1.722	$1.64 \times 10^{-01} \pm 6.66 \times 10^{-02}$	$3.80 \times 10^{-01} \pm 3.15 \times 10^{-01}$	↑
<b>Organic acids</b>				
Formate	8.459	$3.35 \times 10^{-02} \pm 7.96 \times 10^{-02}$	$4.50 \times 10^{-01} \pm 6.88 \times 10^{-01}$	↑
Malonate	3.134	$1.74 \times 10^{-02} \pm 7.29 \times 10^{-03}$	$4.98 \times 10^{-02} \pm 2.56 \times 10^{-02}$	↑
Succinate	2.409	$1.91 \times 10^{-01} \pm 1.63 \times 10^{-01}$	$9.70 \times 10^{-01} \pm 1.12 \times 10^{+00}$	↑
Pyruvate	2.380	$1.22 \times 10^{-01} \pm 1.48 \times 10^{-01}$	$3.38 \times 10^{-01} \pm 3.91 \times 10^{-01}$	↑
Acetate	1.916	$7.24 \times 10^{-01} \pm 5.33 \times 10^{-01}$	$4.73 \times 10^{+00} \pm 3.12 \times 10^{+00}$	↑
<b>SCFAs</b>				
Propionate	2.200	$1.56 \times 10^{-02} \pm 9.42 \times 10^{-03}$	$4.21 \times 10^{-01} \pm 7.61 \times 10^{-01}$	↑
Butyrate	2.150	$5.03 \times 10^{-02} \pm 1.95 \times 10^{-02}$	$1.62 \times 10^{-01} \pm 2.68 \times 10^{-01}$	↑
2-Hydroxyisovalerate	0.837	$2.01 \times 10^{-02} \pm 1.02 \times 10^{-02}$	$3.87 \times 10^{-02} \pm 2.56 \times 10^{-02}$	↑
<b>Amino acids</b>				
Tryptophan	7.749	$1.25 \times 10^{-02} \pm 5.95 \times 10^{-03}$	$9.20 \times 10^{-03} \pm 6.66 \times 10^{-03}$	↓
Phenylalanine	7.391	$9.46 \times 10^{-02} \pm 3.15 \times 10^{-02}$	$6.80 \times 10^{-02} \pm 3.43 \times 10^{-02}$	↓
Tyrosine	7.191	$6.77 \times 10^{-02} \pm 2.53 \times 10^{-02}$	$4.54 \times 10^{-02} \pm 3.51 \times 10^{-02}$	↓
Glutamate	2.354	$3.07 \times 10^{-01} \pm 1.28 \times 10^{-01}$	$1.77 \times 10^{-01} \pm 8.86 \times 10^{-02}$	↓

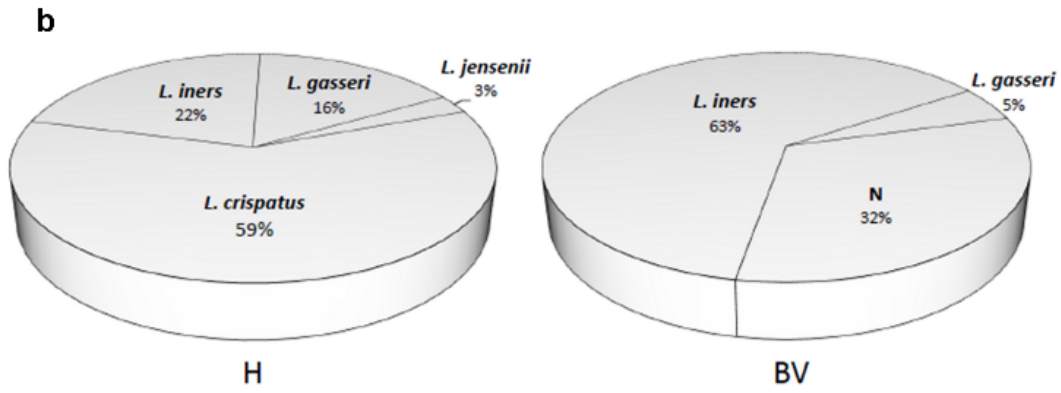
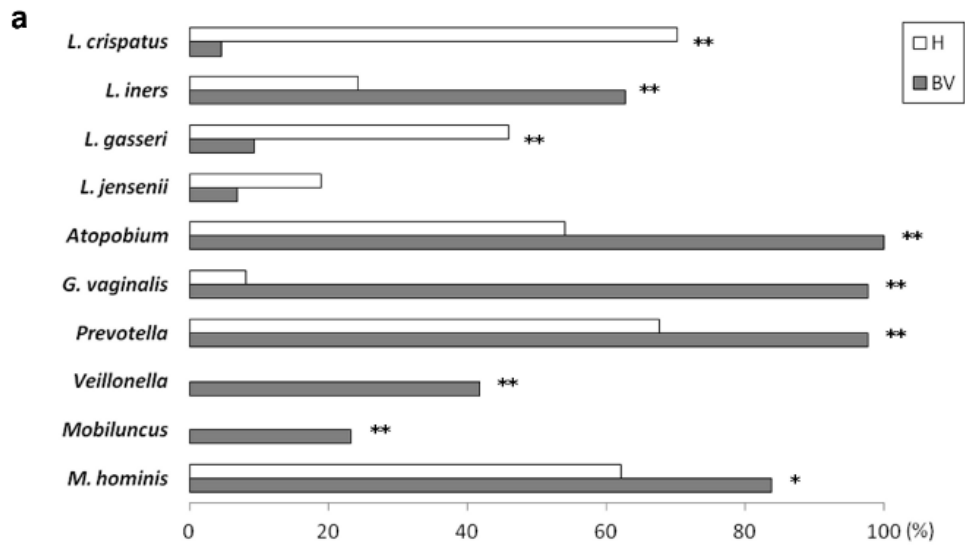
Proline	2.017	$2.95 \times 10^{-02} \pm 1.19 \times 10^{-02}$	$4.70 \times 10^{-02} \pm 2.88 \times 10^{-02}$	↑
Isoleucine	1.012	$8.27 \times 10^{-02} \pm 3.54 \times 10^{-02}$	$6.91 \times 10^{-02} \pm 8.39 \times 10^{-02}$	↓
Leucine	0.962	$4.65 \times 10^{-01} \pm 1.95 \times 10^{-01}$	$3.66 \times 10^{-01} \pm 3.43 \times 10^{-01}$	↓
<b>Nitrogenous bases</b>				
NAD+	9.341	$2.22 \times 10^{-03} \pm 1.58 \times 10^{-03}$	$1.83 \times 10^{-04} \pm 1.28 \times 10^{-03}$	↓
Nicotinate	8.943	$1.63 \times 10^{-03} \pm 1.30 \times 10^{-03}$	$4.77 \times 10^{-03} \pm 1.92 \times 10^{-03}$	↑
Uracil	7.543	$1.32 \times 10^{-02} \pm 6.01 \times 10^{-03}$	$3.12 \times 10^{-02} \pm 3.06 \times 10^{-02}$	↑
Inosine	6.098	$3.61 \times 10^{-03} \pm 7.46 \times 10^{-03}$	$8.12 \times 10^{-04} \pm 1.48 \times 10^{-03}$	↓
<b>Sugars</b>				
Glucose	3.537	$8.27 \times 10^{-02} \pm 6.48 \times 10^{-02}$	$1.67 \times 10^{-01} \pm 1.28 \times 10^{-01}$	↑
Maltose	3.298	$1.17 \times 10^{+00} \pm 6.51 \times 10^{-01}$	$6.27 \times 10^{-01} \pm 5.10 \times 10^{-01}$	↓
<b>Others</b>				
Urocanate	6.410	$6.15 \times 10^{-04} \pm 1.45 \times 10^{-03}$	$3.84 \times 10^{-03} \pm 4.07 \times 10^{-03}$	↑
Kynurenine	6.812	$5.72 \times 10^{-02} \pm 2.33 \times 10^{-02}$	$1.94 \times 10^{-02} \pm 1.84 \times 10^{-02}$	↓
sn-Glycero-3-phosphocholine	3.229	$2.45 \times 10^{-02} \pm 1.11 \times 10^{-02}$	$1.35 \times 10^{-02} \pm 1.72 \times 10^{-02}$	↓
Sarcosine	2.757	$2.99 \times 10^{-02} \pm 1.78 \times 10^{-02}$	$1.17 \times 10^{-02} \pm 6.89 \times 10^{-03}$	↓
2-Amino adipate	2.241	$8.66 \times 10^{-02} \pm 6.09 \times 10^{-02}$	$2.58 \times 10^{-01} \pm 1.55 \times 10^{-01}$	↑
3-Methyl-2-oxovalerate	1.109	$3.34 \times 10^{-03} \pm 3.39 \times 10^{-03}$	$1.06 \times 10^{-02} \pm 1.17 \times 10^{-02}$	↑

532 Values are expressed as means of mmol/l  $\pm$  standard deviation.

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

534

535 Figure 1  
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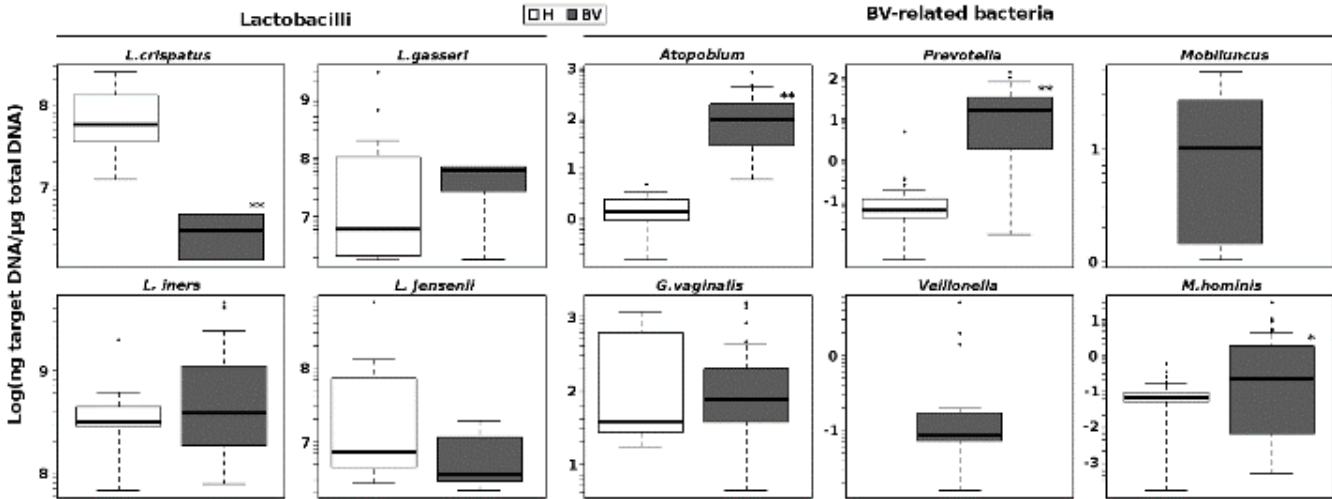


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540 Figure 2

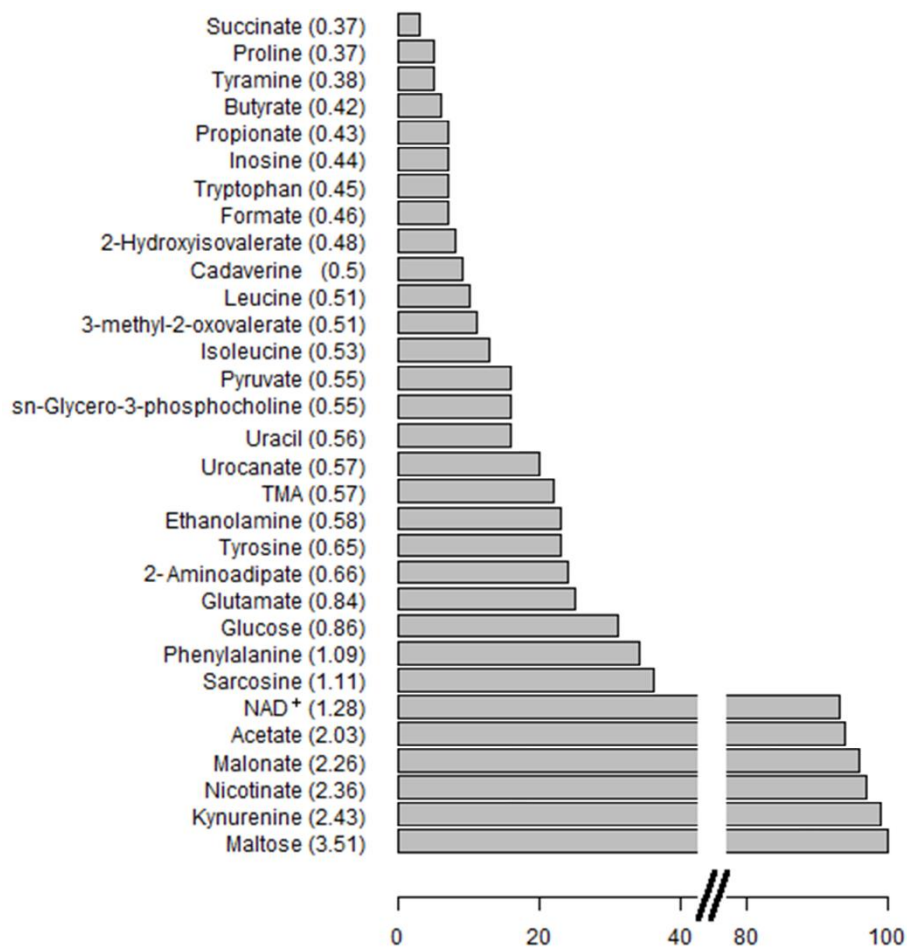


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543 Figure 3

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