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1 **Title: An optimized 16S rRNA sequencing protocol for vaginal microbiome to avoid
2 biased abundance estimation**

3 **Running title:** Optimized primer for vaginal microbiome

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14 **Abstract**

15 We applied three 16S rRNA sequencing protocols on vaginal microbiome samples, to
16 evaluate whether they produce unbiased estimation of vaginal microbiome composition. We
17 modified the 27F primer (hereafter denoted as 27F'). Using vaginal samples from 28 healthy
18 women and 10 women with bacterial vaginosis, we sequenced three 16S rRNA sequencing
19 protocols, i.e., 27F-338R, 27F'-338R and 341F-806R protocols, naming after their PCR
20 primer sets, to test whether the sequencing results are consistent with the clinical diagnostics,
21 morphology and qPCR results. First, the 27F primer would not align with *Gardnerlla*
22 *vaginalis* very well, leading to poor amplification of such species. By modifying the primer
23 sequences, the modified 27F primer (27F') was able to amplify *Gardnerlla vaginalis* very
24 well. Second, the DNA sequence of characteristic species *Lactobacillus crispatus* is identical
25 with *Lactobacillus garrinarum*, leading to biased estimation of abundance of *Lactobacillus*
26 *crispatus* when using V3-V4 as PCR target region; in contrast, such bias did not occur when
27 using V1-V2 as a target region. Third, optimized 27F'-338R avoided above-mentioned biases
28 and restored the well-established community state types (CSTs) clustering.

29

30 **Importance**

31 Vaginal microbiome has profound effects on the health of women and their newborns. Our
32 study found that two well-established 16S rDNA sequencing protocols led to systementical
33 biased estimation of characteristic species of vaginal microbiome. Subsequent analysis
34 proved that the PCR primer fetching efficacy and target region identity were major
35 contributor for such bias. With carefully selected target region and optimized PCR primer set,
36 we were able to eliminate such biases and provide accurate estimation of vaginal
37 microbiome, which showed high consistency with clinical diagnostics. We modified the 27F
38 primer (27F'). Using the optimized PCR primer set of 27F' and 338R to target the V1-V2

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39 hyper-variable region, our 16S rRNA sequencing correctly evaluate the composition of
40 vaginal microbiome.
41
42 **KeyWords**: Bacterial vaginosis; Vaginal microbiome; Primer; 16S rRNA gene hypervariable
43 regions.
44

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45 **Introduction:**

46 The vaginal microbiome has been recognized as a critical factor involved in the protection of
47 the female from various bacterial, fungal and viral pathogens.(1) Bacterial vaginosis (BV) is
48 the most common lower reproductive tract infectious disease in reproductive age women. It is
49 associated with a range of health issues such as pelvic inflammatory disease,(2-4)
50 infertility,(5) preterm delivery,(6) tumors(7, 8) and sexually transmitted diseases.(9-11)
51 Vaginitis was previously diagnosed by culturing bacteria in the vagina, which may overlook
52 some fastidious bacteria that have not been isolated by culture.(12) Nowadays, the diagnosis
53 of BV is typically made by Amsel criteria(13) or Nugent score.(14)

54 With the advent of high-throughput sequencing methods, more and more studies have
55 proposed 16S rRNA sequencing to estimate the composition of vaginal microbiome.(15-17)
56 Partial amplification of bacterial 16S gene sequences with primers across hypervariable
57 regions, mainly including V1-V2 region(15, 18) and V3-V4 region,(17, 19, 20) is a common
58 method to describe vaginal bacterial populations. However, it has been shown that different
59 selection of primers for amplification can bias the results of 16S amplicons for microbiome
60 studies.(21) For example, it has been reported that the universal bacterial 27F primer (5'-
61 AGAGTTTGATCCTGGCTCAG-3') is not suitable for targeting vaginal bacteria in BV such
62 as *Gardnerella vaginalis*.(22) Thus the V1-V2 region primers (27F-338R) did not efficiently
63 evaluate the microbiome in BV.(23)

64 Based on the above research, we modified the sequence of the 27F primer (hereafter
65 denoted as 27F'). And we sequenced three 16S rRNA sequencing protocols, i.e., 27F'-338R,
66 27F-338Rand 341F-806R protocols, naming after their PCR primer sets, to test which
67 provides the best species-level resolution of the vaginal microbiome by means of *in silico*
68 analysis and experimental evaluation.

69

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70

71 ## Results

72 **27F-338R and 341F-805R 16S rRNA protocols could not estimate female vaginal**
73 **microbiome accurately.**

74 We first checked whether the widely used 27F-338R and 341F-805R 16S rRNA protocols
75 were capable of evaluating the vaginal microbiome from women accuratly. 16S rRNA
76 sequencing was applied on the collected vaginal swab samples from 28 healthy women and
77 10 women with BV. As shown in **Table 1**, the top 10 bacteria that showed highest
78 abundance across all the samples were denoted as the representative bacteria of vaginal
79 microbiome. For each sample, any representative bacteria with abundance over 10% was
80 denoted as a major species (highlighted in bold and italic) and others are labeled not detected
81 (ND).

82 First, the abundance of *Gardnerella vaginalis* showed a significant difference between
83 27F-338R and 341F-805R protocols: in the 27F-338R protocol, only 2 out of 10 BV samples
84 (20%) showed *Gardnerella vaginalis* as a major species, while in 341F-805R protocol, 10 out
85 of 10 BV samples (100%) showed *Gardnerella vaginalis*. *Gardnerella vaginalis* was
86 confirmed by morphology and microscope results in all the BV samples (**Appendix Figure**
87 **1**), thus the 341F-805R protocol is more accurate in women. What's more, with *Lactobacilli*
88 and *Gardnerella vaginalis* specific primers, our qPCR validation from 15 random samples
89 also supported the results of 341F-805R protocol (**Appendix Figure 2**).

90 It was also noted that another unexpected bacterium, *Lactobacillus gallinarum*, showed
91 up as a major species in 12 out of 28 healthy samples (43%) from the 341F-805R protocol
92 results. In contrast, no samples showed *Lactobacillus gallinarum* are from the 27F-338R
93 protocol results. To our knowledge, unlike *Lactobacillus crispatus*, *Lactobacillus gasseri*,
94 *Lactobacillus iners*, and *Lactobacillus jensenii*, *Lactobacillus gallinarum* is not a common

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95 *Lactobacilli* in vaginal microbiome.(15) We reasoned that the differences between 16S rRNA
96 protocol may be responsible for such controversial results regarding *Gardnerella vaginalis*
97 and *Lactobacillus gallinarum*.
98
99 **Biased abundance estimations were caused by low fetching efficacy of primer 27F and**
100 **identical sequences in the V3-V4 target region.**
101 We quantified the differences between the 27F-338R and 341F-805R 16S rRNA protocols by
102 the fetching efficacy of primer set and the identity of target regions. To do so, we evaluated
103 the alignments of primer set and target region to the reference databases. To eliminate the
104 potential bias caused by certain reference database, we tested two databases in parallel, i.e.,
105 SLIVA and NCBI 16S Microbioal database.
106 First, we aligned the PCR primer sequences of 27F, 338R, 341F and 805R to the
107 reference 16S rRNA sequence databases to evaluate the primer fetching efficacy. As shown
108 in **Figure 1A**, 27F primer could not align all of the reference sequences (88.9% in SLIVA
109 database and 57.3% in NCBI 16S Microbioal database), compared to 100% for 338R, 341F
110 and 805R primers (in both databases). Two species, i.e., *Gardnerella vaginalis* and
111 *Bifidobacterium bifidum*, were found unable to align with the 27F primer. Another human
112 vaginal microbiome characteristic species, *Atopobium vaginae*, was also found imperfect
113 match with the 27F primer. This is consistent with a previous work that argued 27F primer
114 could reduce PCR efficiency.(22) This also explained why the *Gardnerella vaginalis* was
115 negligible in low abundance from the 27F-338R protocol results.
116 Second, we extracted the target regions corresponding to primer sets of 27F-338R and
117 341F-805R (V1-V2 and V3-V4, correspondingly) and count the identical sequences shared
118 by different species. As shown in **Figure 1B**, there were much more species that share
119 identical sequences with others in the target region of 341F-805R protocol (1062 for SLIVA

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120 database, 747 for NCBI 16S Microbioal database and 543 for intersection of the two
121 databases) than 27F-338R protocol (36 for SLIVA database, 16 for NCBI 16S Microbioal
122 database and 0 for intersection of the two databases). We further checked the species that
123 share identical sequences with others, and found that *Lactobacillus crispatus* share identical
124 sequence with *Lactobacillus gallinarum*, in the target region of 341F-805R primer set
125 (**Figure 1C**). This explained why *Lactobacillus gallinarum* showed in high abundance from
126 the 341F-806R protocol results.

127 To optimize the 16S rRNA protocol, we modified the sequence of 27F primer (see
128 **Methods** for details), to allow higher PCR fetching efficacy. The modified 27F primer was
129 denoted as 27F' and the corresponding 16S protocol was named as 27F'-338R protocol. As
130 shown in **Figure 1A**, in the SLIVA and NCBI 16S Microbioal databases, the 27F' primer
131 aligned 92.6% and 63.4% of reference 16S rRNA sequences, correspondingly; higher than
132 the alignment rate of 27F (88.9% and 57.3%, correspondingly). What's more, the 27F'
133 primer showed perfect match with *Gardnerella vaginalis*, *Bifidobacterium bifidum* and
134 *Atopobium vaginae*. In addition, as shown in **Figure 1B**, 27F'-338R protocol showed 24, -10
135 and 0 species that share identical sequences with others in the target region, from reference
136 database of SLIVA, NCBI 16S Microbioal database and intersection of the two databases,
137 correspondingly. These results indicating that our optimized 27F'-338R 16S rRNA protocol
138 could be a better choice for human vaginal microbiome.

139

140 **Optimized 27F'-338R 16S rRNA protocol provided unbiased estimation of vaginal**
141 **microbiome**

142 We furthur validated the 27F'-338R protocol. First, we merged all the BV samples to count
143 the abundance of the top ten bacteria for three 16S protocols (**Figure 2A**). The top 10 species
144 found in BV condition included *Gardnerella vaginalis, Prevotella* spp., *Lactobacillus iners,*

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145 *Veillonellaceae bacterium, Sneathia amnii, Clostridiales bacterium, Atopobium vaginae,*
146 *Chlamydia trachomatis, Sneathia sanguinegens and Candidatus saccharibacteria*. Overall,
147 we noticed that the results from 27F'-338R and 341F-806R protocols were quite similar and
148 the 27F-338R protocol seemed quite different. The *Gardnerella vaginalis*'s relative
149 abundance is about 41%, 33% and 8%, when applying the 27F'-338R and 341F-806R and
150 27F-338R protocols, respectively. This indicated that the low *Gardnerella vaginalis*
151 estimation from 27F-338R protocol was recalibrated by the 27F'-338R protocol. Second, we
152 merged all the healthy samples to count the abundance of top bacteria under different
153 protocols (**Figure 2B**). Unlike the BV group, the top species were mainly *Lactobacilli,* i.e.,
154 *Lactobacillus crispatus, Lactobacillus iners, Lactobacillus jensenii, Lactobacillus gasseri,*
155 *Lactobacillus gallinarum, Gardnerella vaginalis, Prevotella* spp., *Lactobacillus helveticus,*
156 *Lactobacillus acidophilus and Streptococcus anginosus.* At this time, we noticed that the
157 27F'-338R and 27F-338R protocols were quite similar and the 341F-806R protocol seemed
158 quite different from others. The emerging of in-relevant *Lactobacillus* spp., i.e, *Lactobacillus*
159 *gallinarum*, *Lactobacillus helveticus* and *Lactobacillus acidophilus* in the 341F-806 protocol
160 is because of misalignment due to the identical sequence in the target region. In conclusion,
161 we showed that the 27F'-338R protocol could recalibrate the biased estimation of
162 *Gardnerella vaginalis* and *Lactobacillus crisptus*.

163 Subsequently, we found the 27F'-338R protocol could restore the well-established
164 community state types (CSTs) clustering.(15) We performed unsupervised clustering of 28
165 healthy and 10 BV samples using the abundance of the top 20 bacteria (**Figure 3**). We
166 noticed all the healthy samples were clustered together and all the BV samples were clustered
167 together. All the BV samples showed *Lactobacillus* diminished and *Gardnerella vaginalis*
168 dominated diverse community, similar to the CST-IV cluster.(15) For the healthy samples,
169 we noticed all *Lactobacillus crispatus* enriched samples were clustered together, so were the

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170 *Lactobacillus gasseri* enriched samples, the *Lactobacillus iners* enriched samples and the
171 *Lactobacillus iners* enriched samples; and they formed the CST-I, CST-II, CST-III and CST-
172 V cluster.(15) In summary, we propose that the 27F'-338R protocol based 16S rRNA
173 sequencing method could give an unbiased estimation of vaginal microbiome.
174

175 ## Disscussion

176 16S rRNA sequencing has been used to identify the bacterial composition of the human
177 vaginal microbiome in multiple ethnic groups, but the study on the population's vaginal
178 microbiome is still insufficient. In addition, no studies have examined whether different 16S
179 rRNA sequencing protocols are an unbiased way to identify vaginal microbes. Our principal
180 findings were that the 27F primer was not well aligned with *Gardnerlla vaginalis*, resulting
181 in poor amplification effect. By modifying the 27F primer, 27F' could well amplify
182 *Gardnerlla vaginalis*; The DNA sequence of *Lactobacillus crispatus* was the same as that of
183 *Lactobacillus garrinarum*. There was a bias in the estimation of *Lactobacillus crispatus*
184 abundance when V3-V4 was the target region of PCR, while there was no such bias when
185 V1-V2 was the target region; The optimized 27F '-338R avoids the above deviation and
186 restores the well-established community state types (CSTs) clustering.

187 As we showed in the introduction section, a series of 16S rRNA sequencing protocols
188 with different target regions and corresponded primer sets were utilized in vaginal
189 microbiome studies. However, due to the limit on reads length, only a subset of target regions
190 remains available. One recent study had performed in-silico and experimental evalutions on
191 primer sets of V1-V3, V3-V4 and V4. In their conclusion, V4 region provides the best results
192 on species level resolution of the vaginal microbiome.(21) In our evaluation, we emphasized
193 the consistency between the 16S rRNA sequencing results and clinical diagnosis, such as
194 morphology and culture of the characteristic species. Another study compared two 16S rRNA

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195 protocols, utilizing V1-V2 and V3-V4 hypervariable regions as target regions. They found
196 16S rRNA sequencing protocol utilizing V3-V4 hypervariable region would identified more
197 species and the ones using V1-V2 hypervariable region would miss several characteristic
198 speices of vaginal microbiome.(23) We agreed with them that unoptimized 16S rRNA
199 sequencing protocol utilizing V1-V2 hypervariable region would produce biased estimation.
200 *Gardnerella vaginalis* is a well recognized bacteria, which is confirmed by
201 morphology and microscope results in all the BV samples. However, through our *in-silico*
202 analysis, *Gardnerella vaginalis* were found unable to align with the 27F primer. This is
203 consistent with previous reports as the 27F primer could not match the *Gardnerella vaginalis*
204 very well, leading to a low PCR efficiency. [22] For other microbiome, if we normalized the
205 *Gardnerella vaginalis*'s abundance, they showed no significant difference under the 27F'-
206 338R and 341F-806R and 27F-338R protocols.
207 *Lactobacillus* spp. are so important in human vaginal microbiome that four
208 *Lactobacillus* spp. were the characteristic species used by the authoritative five community
209 state types (CSTs), which are established to group vaginal microbiome patterns according to
210 the dominant species present: CSTI, II, III, IV and V dominated by *L.crispatus*, *L. gasseri*, *L.*
211 *iners*, diverse community and *L. jensenii*, respectively.(15) However, we found that
212 *Lactobacillus crispatus* share identical sequence with *Lactobacillus gallinarum* when using
213 the target region of 341F-805R primer set. That is, if we used the V3-V4 as the target region,
214 we might wrongly assign the characteristic species of CST-I (*Lactobacillus crispatus*) to
215 another vaginal microbiome in-relevant species (*Lactobacillus gallinarum*).
216 As shown in our trial experiments, the 27F-338R protocol under-estimated the
217 abundance of *Gardnerella vaginalis*. In addition, we showed that 16S rRNA sequencing
218 protocol utilizing V3-V4 hypervariable region would also introduce bias: the 341F-806R
219 protocol misaligned *Lactobacillus crisptus* to other in-relevant *Lactobacilli*. What's more,

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220 these biases only occurs in its own protocol, but could not be repeated in the other protocol.
221 Therefore, we reasoned that such bias was not sample or ethnic group related, but instead,
222 associated with unoptimized 16S rRNA sequencing protocols. We have pinned down that
223 primer sequence and target region are the major contributor for the bias. Subsequently, we
224 have optimized the protocol, using the modified 27F primer and chose the V1-V2 hyper-
225 variable region as the target region. The optimized 16S rRNA sequencing protocol had been
226 proven to be able to recalibrate the estimation of *Gardnerella vaginalis,* preventing
227 misalignment of *Lactobacillus crispatus* and restored the authoritative five community state
228 types (CSTs).

229 This study provides an optimized 16S rRNA-based protocol for evaluating the
230 composition of human vaginal microbiome using current common NGS sequencing platform.
231 and it is the first piece of work that systematically investigated the female vaginal
232 microbiome with above-mentioned methods. This optimized 16S rRNA-based protocol can
233 not only accurately assess the composition of vaginal flora, but also accurately and
234 economically. The accurate assessment of vaginal microbiome could contribute to the
235 treatment of vaginitis in hospital.

236 Serval further works will be updated regard the following aspects. In this study, we
237 used BV sample and healthy samples, because the vaginal microbiome is mainly dominated
238 by bacteria in these two groups. Another bacterium dominate disease, aerobic vaginitis, will
239 be tested in our subsequent work. Yet, one disadvantage of the 16S rRNA sequencing was
240 exposed, as well and that is that the 16S rRNA sequencing is not suitable for the diagnosis of
241 TV, VVC, HPV, HIV and so on. Currently, we used the clinical diagnostics such as such as
242 morphology and culture of the characteristic species as ground truth of human vaginal
243 microbiome's composition. However, the composition of human vaginal microbiome is
244 constantly being updated as more and more new technologies are being applied, such as

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245 metagenome related technology. It should also be noted, that as we were restricted by the
246 sequencing platform, we only tested the target regions of the V1-V2 and V3-V4, leaving the
247 V1-V3, V4, V4-V6 target regions unexamined, albeit future work will examine such target
248 regions not included in the present study.
249

250 Materials and methods

251 27F' primer design

252 As mentioned above, the common nondegenerate form of the 27F primer (5'-
253 AG**A**GTT**T**GAT**C**CTGGCTCAG-3') is not suitable for targeting *Gardnerella vaginalis* in
254 BV.(22) Meanwhile, the sequence (5'-AG**G**GTT**C**GAT**T**CTGGCTCAG-3') most frequently
255 observed binding site sequence is found in *Bifidobacteriales*, including the genus
256 *Gardnerella* (GenBank accession numbers M58729 to M58744).(22, 24) Its binding site
257 variant is of particular interest to the study of vaginal microbiology in BV, and the sequence
258 has three mismatched bases compared to the common sequence of the 27F primer. To
259 combine two sequences' strengths, we merged their different bases (R=A/G,Y=T/C), and got
260 an modified 27F primer, i.e., 27F' (5'-AG**R**GTT**Y**GAT**Y**CTGGCTCAG-3').
261

262 Study Population:

263 28 healthy women without vaginitis such as aerobic vaginitis (AV), bacterial vaginosis (BV),
264 vulvovaginal candidiasis (VVC), and trichomonas vaginitis (TV), and 10 women with BV
265 only were enrolled at the gynecological clinic of Beijing Tsinghua Changgung Hospital from
266 April to October 2018. All women were aging between 18 and 50 years old and were not
267 pregnant or breast-feeding. The protocol was approved by the Medical Ethics Committee of
268 Beijing Tsinghua Changgung Hospital. Written informed consents were obtained from each
269 participant.

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270

271 **Sample collection and DNA Extraction**

272 The vaginal secretions were obtained via two swabs. One swab was used to prepare a dry
273 slide for Gram staining, under 400× magnification for visual detection, to test for AV, BV,
274 VVC, and TV. The criteria of Donders(25) et al. was used to diagnose AV (with a score of 3
275 or greater). BV was determined by Nugent's criteria (Nugent score of 7 or greater).(14) The
276 diagnosis of VVC and TV was mainly based on morphological observation under high power
277 field (400× magnification). The other swab was quickly plunged into a tube containing 1 ml
278 PBS solution and stored at -80℃ until total DNA extraction of vaginal flora. The DNA of the
279 sample was extracted through the TIANamp Bacteria DNA Kit (TIANGEN, China)
280 according to the manufacturer's instructions. This step required additional Lysozyme (Sigma–
281 Aldrich), proteinase K, RNase A (Sigma–Aldrich), and finally washed and stored the DNA
282 with 1×TE buffer. A spectrophotometer was used (Thermo Scientific NanoDrop One) to
283 measure the concentration and purity of the DNA extracts. Then isolated DNA was stored at -
284 20℃ until needed.

285

286 **Sequencing**

287 Taking data volume, sequencing accuracy, read length and economic factors into account, in
288 this study, we chose the pair-end Illumina Solexa sequencing platform over 454
289 pyrosequencing platform. The V1-V2 and V3-V4 regions of the 16S rRNA were then
290 separately amplified with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3')
291 and 338R (5'-GCTGCCTCCCGTAGGAGT-3'), 341F (5'-CCTAYGGGRBGCASCAG-3')
292 and 806R (5'-GGACTACNNGGGTATCTAAT-3'). The V1-V2 regions were also amplified
293 with our modified primers 27F' (5'-AGRGTTYGATYCTGGCTCAG-3') and 338R (5'-
294 GCTGCCTCCCGTAGGAGT-3'). All PCR reactions were carried out with Phusion® High-

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295 Fidelity PCR MasterMix (New England Biolabs). The PCR products examined with 400-
296 450bp were chosen and mixed in equal density ratios. Then, the mixture PCR product was
297 purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were
298 generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA)
299 following the manufacturer's recommendations and index codes were added. The library
300 quality was assessed on the Qubit@ 2.0Fluorometer (Thermo Scientific) and Agilent
301 Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq 2500
302 platform and 250 bp paired-end reads were generated.

303

304 **Reference Database**

305 We compared SLIVA and NCBI in the following evaluations, as the Green genes database
306 has not been updated since 2013(26) and RDP database is semi-automatic curated.(27) For
307 the SLIVA database, we used and downloaded the SSU 128 Ref NR 99 version from
308 https://www.arb-silva.de. For the NCBI database, we downloaded using the blast command
309 of blastdbcmd in June 2017. All the taxonomies are summarized into species level.

310

311 **Sequencing Data Processing**

312 Paired-end reads were assigned to samples according to the sample-specific barcode and
313 truncated by cutting off the barcode and primer sequence. Use the software
314 FLASH(V1.2.7)(28) to merge paired-end reads. According to the QIIME(V1.7.0)(29) quality
315 control process, the raw tags were mass filtered under specific filtration conditions to obtain
316 high quality clean tags.(30)

317 The 16S sequence reference index was built using the command “bowtie2-build”,
318 with default parameters. All reads were aligned against the prebuild index using bowtie2,
319 with parameter of “bowtie2 --local”. Alignments were associated to taxonomy by a sequence-

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320 id-to-taxonomy map, provided by the reference database, using a custom Perl script. Unique
321 reads were counted for each taxonomy and abundance was calculated for all taxonomy.
322 Species with abundance lower than 1% or reads number less than 5 were excluded.
323
324 **qPCR validation**
325 *Lactobacilli* and *Gardnerella vaginalis* specific qPCR primer and probe sequences were
326 synthesized as previously described.(31) DNA was amplified using SGExcel GoldStar
327 TaqMan qPCR Mix (Sangon Biotech) on a Bio-Rad CFX96 real-time PCR detection system.
328
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336

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433 ## Figure Legends

434 **Figure 1:** PCR primer fetching efficacy and target region identity quantification.

435 A. Primer efficiency were quantified by the alignment of primer sequence to the reference
436 sequences. In X-axis, two reference databases were used, SLIVA and NCBI 16S
437 Microbioal. The Y-axis showed the percentage of aligned reference sequences by certain
438 primer sequences, including 27F' (blue), 27F (orange), 338R (grey), 341F (yellow) and
439 805R (dark blue).

440 B. Number of identical sequences shared by two different species had been shown in bar
441 plot. The X-axis represents the reference database we used.

442 C. Alignment of *Lactobacillus crispatus* and *Lactobacillus gallinarum* at V3-V4 region.

443

444 **Figure 2:** Comparison of 16S rRNA sequencing results from 27F-338R, 27F'-338R and
445 341F-806R protocols.

446 A. The top ten bacteria's abundance were from the BV group. Three protocols were
447 compared, i.e., 27F-338R (blue), 27F'-338R (orange) and 341F-806R (grey).

448 B. Like in A, the top ten bacteria showed in the healthy group from three protocols, i.e., 27F-
449 338R (blue), 27F'-338R (orange) and 341F-806R (grey), were compared.

450

451 **Figure 3:** Heatmap and dendrogram of vaginal compositions from 28 healthy and 10 BV
452 samples.

453 The vaginal compositions from 28 healthy and 10 BV samples utilizing 27F'-338R protocol
454 were clustered and colored by relative abundance (from low to high abundance, color
455 changes from green to red).

456

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457 **Appendix Figure 1**: Morphology of samples under 400× magnification after gram staining.

458 A: 28 normal samples, B: 10 BV samples.

459

460 **Appendix Figure 2**: qPCR validation of the existence of Lactobacilli and Gardnerella

461 vaginalis.

462 10 vaginal microbiome samples from healthy women (highlighted in blue) and 5 from

463 women with BV (highlighted in orange) were sampled and used to perform qPCR validation.

464 The difference between the Cq values of *Lactobacilli* and *Gardnerella vaginalis* was used.

465

466

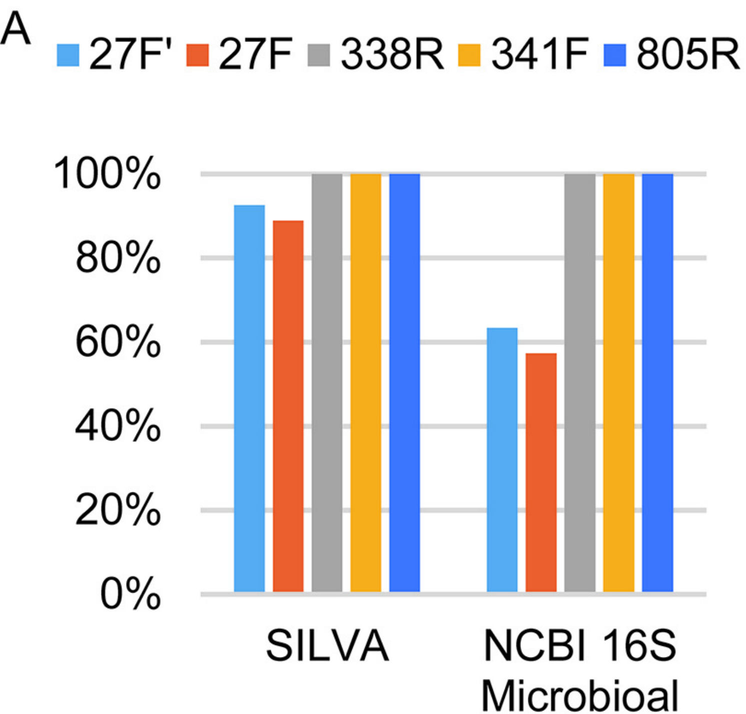
Table 1. Summary of vaginal microbiome compositions from healthy and BV samples.

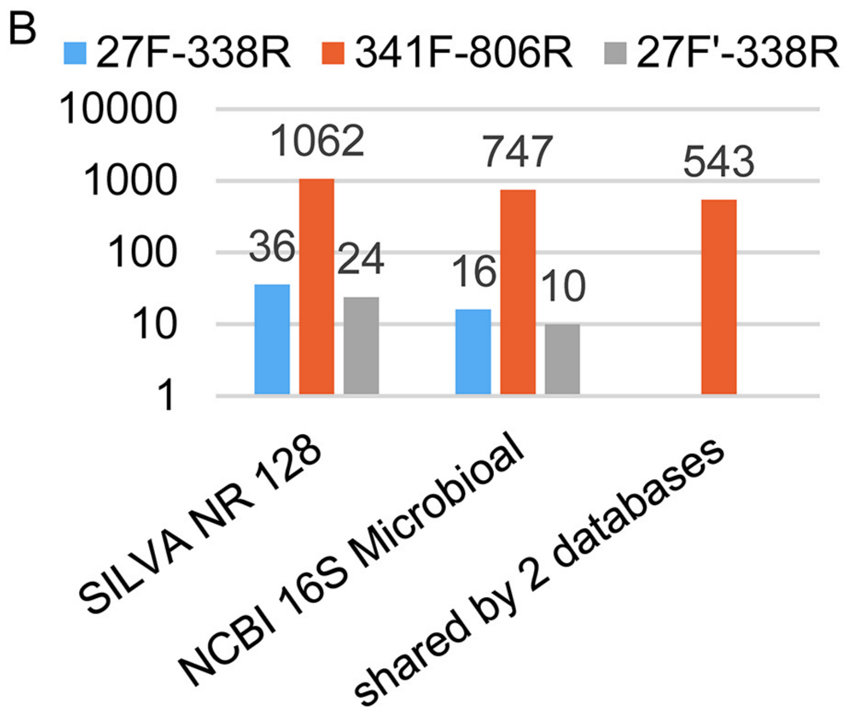
Sample ID	*L. crispatus*		*L. iners*		*L. jensenii*		*L. gasseri*		*L. gallinarum*		*P. spp*		*G. vaginalis*		*A. vaginae*		*V. bacterium*		*S. amnii*	
	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R
1	***98%***	***77%***	ND	ND	ND	ND	ND	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	96%	94%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	***98%***	***75%***	ND	ND	ND	ND	ND	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	***98%***	***82%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	***97%***	***77%***	ND	ND	ND	ND	ND	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	***72%***	***60%***	ND	ND	ND	ND	ND	ND	ND	***10%***	***25%***	***20%***	ND	ND	ND	ND	ND	ND	ND	ND
7	***95%***	***59%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	***96%***	***61%***	ND	ND	ND	ND	ND	ND	ND	***11%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9	***45%***	***40%***	***52%***	***48%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10	***79%***	***65%***	***16%***	***17%***	ND	ND	ND	ND	ND	***11%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	ND	ND	***97%***	***94%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
12	***94%***	***77%***	ND	ND	ND	ND	ND	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
13	ND	ND	***95%***	***93%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	***97%***	***76%***	ND	ND	ND	ND	ND	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15	***98%***	***79%***	ND	ND	ND	ND	ND	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16	ND	ND	***95%***	***89%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
17	ND	ND	ND	ND	ND	ND	***95%***	***96%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	***97%***	***79%***	ND	ND	ND	ND	ND	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
19	ND	ND	***95%***	***96%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
20	ND	ND	***96%***	***95%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Sample ID	*L. crispatus*		*L. iners*		*L. jensenii*		*L. gasseri*		*L. gallinarum*		*P. spp*		*G. vaginalis*		*A. vaginae*		*V. bacterium*		*S. amnii*	
	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R	27F-338R	341F-806R
21	***94%***	***76%***	ND	ND	ND	ND	ND	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
22	ND	ND	ND	ND	***93%***	***90%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
23	***33%***	***27%***	***65%***	***65%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24	***33%***	***31%***	***64%***	***59%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
25	***27%***	***20%***	***68%***	***55%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	***11%***	ND	ND	ND	ND	ND	ND
26	ND	ND	***96%***	***90%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
27	ND	ND	ND	***12%***	***87%***	***43%***	ND	ND	ND	ND	ND	ND	ND	***40%***	ND	ND	ND	ND	ND	ND
28	***93%***	***68%***	ND	ND	ND	ND	ND	ND	ND	***12%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BV1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	***27%***	***24%***	ND	***36%***	***20%***	***10%***	***14%***	***11%***	ND	ND
BV2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	***26%***	***25%***	ND	***31%***	***29%***	***19%***	***17%***	***14%***	ND	ND
BV6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	***22%***	***16%***	ND	***20%***	ND	ND	***11%***	ND	***50%***	***45%***
BV3	ND	ND	ND	***17%***	ND	ND	ND	ND	ND	ND	***30%***	***17%***	ND	***25%***	***13%***	ND	***13%***	ND	ND	ND
BV7	ND	ND	***13%***	ND	ND	ND	ND	ND	ND	ND	***22%***	***19%***	ND	***37%***	***14%***	ND	***21%***	***15%***	ND	ND
BV8	ND	ND	***26%***	***23%***	ND	ND	ND	ND	ND	ND	***17%***	***16%***	ND	***30%***	ND	ND	***20%***	***17%***	ND	ND
BV4	ND	ND	***60%***	***45%***	ND	ND	ND	ND	ND	ND	ND	ND	ND	***29%***	ND	ND	ND	ND	ND	ND
BV5	ND	ND	***41%***	***21%***	ND	ND	ND	ND	ND	ND	***22%***	ND	***16%***	***49%***	ND	ND	ND	ND	ND	ND
BV9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	***42%***	***25%***	***11%***	***44%***	ND	ND	ND	ND	***40%***	***24%***
BV10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	***29%***	***28%***	ND	***38%***	***24%***	***12%***	***18%***	***14%***	ND	ND

Abbreviation: BV, bacterial vaginosis. ND, not detected.

Each row represents a sample ID and each column represents the corresponding relative abundance of a species under a 16S rDNA sequencing protocol. Only the top 10 bacteria that showed highest abundance across all the samples were shown. Abundance higher than 10% is highlighted with italic and bold font, and others are labeled ND.





C

```
Seq_1  1    TAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1    TAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG  60

Seq_1  61   TTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61   TTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTA  120

Seq_1  121  TTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121  TTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG  180

Seq_1  181  TAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAAG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  181  TAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAAG  240

Seq_1  241  TCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTGAGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241  TCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTGAGT  300

Seq_1  301  GCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301  GCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAAC  360

Seq_1  361  ACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361  ACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTA  420

Seq_1  421  GCGAACAG  428
            ||||||||
Seq_2  421  GCGAACAG  428
```

A

Gardnerella_vaginalis
Prevotella_sp
Lactobacillus_iners
Veillonellaceae_bacterium
Sneathia_amnii
Atopobium_vaginae
Clostridiales_bacterium
Chlamydia_trachomatis
Sneathia_sanguinegens
Candidatus_Saccharibacteria

0% 10% 20% 30% 40% 50%

341F-806R 27F'-338R 27F-338R

B

Lactobacillus_crispatus
Lactobacillus_iners
Lactobacillus_jensenii
Lactobacillus_gasseri
Lactobacillus_gallinarum
Gardnerella_vaginalis
Prevotella_sp
Lactobacillus_helveticus
Lactobacillus_acidophilus
Streptococcus_anginosus

0 0.1 0.2 0.3 0.4 0.5 0.6

341F-806R 27F'-338R 27F-338R

Color Key

0 0.2 0.4 0.6 0.8

Value

Lactobacillus crispatus

Lactobacillus iners

Lactobacillus gasseri

Lactobacillus jensenii

Gardnerella vaginalis

Atopobium vaginae

Prevotella sp

Veillonellaceae bacterium

Sneathia amnii

Sneathia sanguinegens

Chlamydia trachomatis

Clostridiales bacterium

Streptococcus anginosus

Candidatus Saccharibacteria

Coriobacteriales bacterium

6 10 28 5 4 15 1 3 21 12 7 14 8 18 17 9 25 24 23 11 26 13 16 2 19 20 22 27 BV5 BV4 BV9 BV6 BV8 BV3 BV7 BV2 BV1 BV10