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6 # Evaluation of DNA extraction protocols from liquid-based cytology specimens
7 # for studying cervical microbiota
8

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24 **Abstract**

25 Cervical microbiota (CM) are considered an important factor affecting the progression of
26 cervical intraepithelial neoplasia (CIN) and are implicated in the persistence of human
27 papillomavirus (HPV). Collection of liquid-based cytology (LBC) samples is routine for cervical
28 cancer screening and HPV genotyping, and can be used for long-term cytological biobanking.
29 Herein, we investigate the feasibility of leveraging LBC specimens for use in CM surveys by
30 amplicon sequencing. As methodological differences in DNA extraction protocols can
31 potentially bias the composition of microbiota, we set out to determine the performance of four
32 commonly used DNA extraction kits (ZymoBIOMICS DNA Miniprep Kit; QIAamp PowerFecal
33 Pro DNA Kit; QIAamp DNA Mini Kit; and IndiSpin Pathogen Kit) and their ability to capture
34 the diversity of CM from LBC specimens. LBC specimens from 20 patients (stored for 716 ±
35 105 days) with cervical intraepithelial neoplasia (CIN) 2/3 or suspected CIN2/3 were each
36 aliquoted for extraction by each of the four kits. We observed that, regardless of the extraction
37 protocol used, all kits provided equivalent accessibility to the cervical microbiome, with some
38 minor differences. For example, the ZymoBIOMICS kit appeared to differentially increase
39 access of several more microbiota compared to the other kits. Potential kit contaminants were
40 observed as well. Approximately 80% microbial genera were shared among all DNA extraction
41 protocols. The variance of microbial composition per individual was larger than that of the DNA
42 extraction protocol used. We also observed that HPV16 infection was significantly associated
43 with community types that were not dominated by *Lactobacillus iners*.

44

45 **Importance**

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46 Collection of LBC specimens is routine for cervical cancer screening and HPV genotyping, and
47 can be used for long-term cytological biobanking. We demonstrated that LBC samples, which
48 had been under prolonged storage prior to DNA extraction, were able to provide a robust
49 assessment of the CM and its relationship to HPV status, regardless of the extraction kit used.
50 Being able to retroactively access the CM from biobanked LBC samples, will allow researchers
51 to better interrogate historical interactions between the CM and its relationship to CIN and HPV.
52 This alone has the potential to bring CM research one-step closer to the clinical practice.

53

54 **Keywords**; cervical microbiota, DNA extraction, HPV, CIN, liquid-based cytology

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55 **Introduction**

56 High-throughput sequencing (HTS) technology of 16S rRNA gene amplicon sequences
57 has made it possible to better understand the relationships between cervicovaginal microbiota
58 and human papillomavirus (HPV) infection (1) (2) (3) (4) (5) and HPV-related diseases (6) (7)
59 (8) (9) (10). Cervicovaginal microbiota are considered to be an important factor affecting the
60 progress of cervical intraepithelial neoplasia (CIN) (6) (7) (8) (9) and are implicated in the
61 persistence of high-risk HPV (HR-HPV) (1) (2) and low-risk HPV (LR-HPV) (3). For example,
62 the phyla *Actinobacteria* and *Fusobacteria* were enriched in HR-HPV positive environment (4)
63 while the phyla *Actinobacteria*, *Proteobacteria*, and *Fusobacteria* in low-risk HPV (LR-HPV)
64 (3). Additionally, *Lactobacillus iners*-dominant samples are associated with both HR-HPV and
65 LR-HPV (5). Moreover, it has been shown that CIN risk was increased when the cervical
66 microbes *Atopobium vaginae*, *Gardnerella vaginalis*, and *Lactobacillus iners* were present with
67 HR-HPV (10). The cervicovaginal microbiome specified by *Lactobacillus*-dominant type or non-
68 *Lactobacillus*-dominant type has been shown to interact with the immune system (7) (11).
69 Inflammatory cytokines, such as Interleukin (IL)-1α and IL-18, were increased in non-
70 *Lactobacillus*-dominant community types of reproductive-aged healthy women (11). In the
71 analysis of patients with cervical cancer, non-*Lactobacillus*-dominant community types were
72 positively associated with chemokines such as interferon gamma-induced protein 10 (IP-10) and
73 soluble CD40-ligand activating dendric cells (DCs) (7). The metabolism of the cervicovaginal
74 microbiome may be a substantial contributing factor to maternal health during pregnancy,
75 although the mechanism is still unclear (12).

76 Little has been reported on the utility of liquid-based cytology (LBC) samples for use in
77 cervical microbiome studies. Conventionally, microbiome sample collection methods entail the

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78 use of swabs (13) or self-collection of vaginal discharge (14). To obtain a non-biased and broad
79 range of cervical microbiota, DNA extraction should be optimized for a range of difficult-to-
80 lyse-bacteria, *e.g*. *Firmicutes*, *Actinobacteria*, and *Lactobacillus* (13) (15) (16) (17) (18).

81 LBC samples are promising for cervicovaginal microbiome surveys, as they are an
82 already established method of long-term cytological biobanking (19). In clinical practice,
83 cervical cytology for cervical cancer screening or HPV genotyping is widely performed using a
84 combination of cervical cytobrushes and LBC samples such as ThinPrep (HOLOGIC) or
85 SurePath (BD). An LBC specimen can be used for not only cytological diagnosis but also
86 additional diagnostic tests such as HPV, *Chlamydia*, *Neisseria gonorrhoeae*, and *Trichomonas*
87 infection (20) (21) (22).

88 The ability to characterize microbial communities, as commonly assessed by 16S rRNA
89 gene sequencing, can be biased as a result of methodological differences of cell lysis and DNA
90 extraction protocols (23) (24) (25). Herein, we compare four different commercially available
91 DNA extraction kits in an effort to assess their ability to characterize the cervical microbiota of
92 LBC samples. Additionally, we examine the relationship between HPV infection and the
93 composition of cervical microbiota.

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94 **Materials and Methods**

95 **Ethics**

96 This study was approved by the Institutional Review Board at the University of Arkansas for
97 Medical Sciences (IRB number 202790).
98

99 **Sampling of cervical microbiome**

100 LBC specimens were obtained from 20 patients enrolled in a Phase II clinical trial of an HPV
101 therapeutic vaccine (NCT02481414). In order to be eligible, participants had to have high grade
102 squamous intraepithelial lesions (HSILs) or cannot rule out HSILs in cervical cytology or
103 CIN2/3 in cervical biopsy. Those who qualified for the study based on their cervical cytology
104 underwent cervical biopsy, and they qualified for vaccination if the results were CIN2/3. The
105 cervical cytology specimens in this current study were collected before the vaccination and
106 reserved in the vial of the ThinPrep Pap Test (HOLOGIC) as described in Ravilla *et al*. 2019
107 (26). The storage period from sample collection to DNA extraction was 716 ± 105 days in this
108 study.
109

110 **HPV genotyping**

111 HPV-DNA was detected by Linear Array HPV Genotyping Test (Roche Diagnostics) which can
112 detect up to 37 HPV genotypes including 13 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51,
113 52, 56, 58, 59, and 68) and 24 LR-HPV genotypes (6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 66,
114 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108) using ThinPrep solution
115 (https://diagnostics.roche.com/global/en/products/params/linear-array-hpv-genotyping.html).
116

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117 **DNA extraction protocols**

118 We selected four commercially available DNA extraction kits as the candidates for comparison:
119 ZymoBIOMICS DNA Miniprep Kit (Zymo Research, D4300), QIAamp PowerFecal Pro DNA
120 Kit (QIAGEN, 51804), QIAamp DNA Mini Kit (QIAGEN, 51304), and IndiSpin Pathogen Kit
121 (Indical Bioscience, SPS4104). These kits have been successfully used in a variety of human
122 cervical, vaginal, and gut microbiome surveys (10) (19) (27). We'll subsequently refer to each of
123 these kits in abbreviated form as follows: ZymoBIOMICS, PowerFecalPro, QIAampMini, and
124 IndiSpin. The protocols and any modifications are outlined in Table 1.

125 Each LBC sample was dispensed into four separate 2 mL sterile collection tubes
126 (dispensed sample volume = 500 μL) to create four cohorts of 20 DNA extractions (Fig. 1). Each
127 extraction cohort was processed through one of the four kits above. A total of 80 extractions (4
128 kits × 20 patients) were prepared for subsequent analyses. Applied sample volume of ThinPrep
129 solution was 300 μL for ZymoBIOMICS, 300 μL for PowerFecalPro, and 200 μL for
130 QIAampMini, and 300 μL for IndiSpin. The sample volume was standardized to 300 μL as long
131 as the manufacturer's instructions allowed to do so. DNA extraction for all samples was
132 performed by the same individual who practiced by performing multiple extractions for each kit
133 before performing the actual DNA extraction on the samples analyzed in this study. Positive
134 control was mock vaginal microbial communities composed of a mixture of genomic DNA from
135 the American Type Culture Collection (ATCC MSA1007). Negative control was the ThinPrep
136 preservation solution without the sample as blank extraction (28).

137

138 **Measurement of DNA yield**

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139 DNA yield for each method was evaluated by spectrophotometer (Nanodrop One, Thermo
140 Scientific). Analysis of the DNA yield from IndiSpin was omitted as nucleic acid is used as a
141 carrier for this kit. The mean DNA yields per 100 μL ThinPrep sample volume were compared.
142

143 **16S rRNA marker gene sequencing**

144 Controls and the extracted DNA were sent to Argonne National Laboratory (IL, USA) for
145 amplification and sequencing of the 16S rRNA gene on an Illumina MiSeq sequencing platform.
146 Paired-end reads from libraries with ~250-bp inserts were generated for the V4 region using the
147 barcoded primer set: 515FB: 5'-GTGYCAGCMGCCGCGGTAA-3' and 806RB: 5'-
148 GGACTACNVGGGTWTCTAAT-3' (29) (30) (31) (32)
149 (http://www.earthmicrobiome.org/protocols-and-standards/16s/). MiSeq Reagent Kit v2 (2 × 150
150 cycles, MS-102-2002) was used.
151

152 **Sequence processing and analysis**

153 Initial sequence processing and analyses were performed using QIIME 2 (33), any commands
154 prefixed by `q2-` are QIIME 2 plugins. After demultiplexing of the paired-end reads by `q2-`
155 `demux`, the imported sequence data was visually inspected via QIIME 2 View
156 (https://view.qiime2.org), to determine the appropriate trimming and truncation parameters for
157 generating Exact Sequence Variants (ESVs) (34) via `q2-dada2` (35). Hereafter, ESVs will be
158 referred to as OTUs (Operational Taxonomic Units). The forward reads were trimmed at 15 bp
159 and truncated at 150 bp; reverse reads were trimmed at 0 bp and truncated at 150 bp. The
160 resulting OTUs were assigned taxonomy through `q2-feature-classifier classify-`
161 `sklearn`, by using a pre-trained classifier for the amplicon region of interest (36). This enables

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162 more robust taxonomic assignment of the OTUs (37). Taxonomy-based filtering was performed
163 by using `q2-taxa filter-table` to remove any OTUs that were classified as
164 "Chloroplast", "Mitochondria", "Eukaryota", "Unclassified" and those that did not have at least
165 a Phylum-level classification. We then performed additional quality filtering via `q2-quality-`
166 `control`, and only retained OTUs that had at least a 90% identity and 90% query alignment to
167 the SILVA reference set (38). Then `q2-alignment` was used to generate a *de novo* alignment
168 with MAFFT (39) which was subsequently masked by setting `max-gap-frequency 1`
169 `min-conservation 0.4`. Finally, q2-phylogeny was used to construct a midpoint-rooted
170 phylogenetic tree using IQ-TREE (40) with automatic model selection using ModelFinder (41).
171 Unless specified, subsequent analyses were performed after removing OTUs with a frequency of
172 less than 0.0005% of the total data set (42).
173

174 **Number of reads and OTUs before rarefying**

175 Table 3 highlights the numbers of reads and OTUs among the DNA extraction protocols prior to
176 rarefying the data. The reads and OTUs assigned to gram-positive and gram-negative was also
177 shown. The number of "OTUs before rarefying" shown in Table 3 is distinguished from the
178 "Observed OTUs" after rarefying in Fig. 3 for diversity analysis.
179

180 **Microbiome analysis**

181 To compare the taxonomic profiles among four types of DNA extraction methods (Fig. 1 &
182 Table 1), the following analyses were performed; (I) bacterial microbiome composition, (II)
183 detection of common and unique taxa, (III) alpha and beta diversity analysis, and (IV)
184 identification of specific bacteria retained per DNA extraction method.

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185

186 **Microbiome composition**

187 We generated the bar plot to exhibit bacterial microbiome composition per DNA extraction
188 method at the family (Fig. 2A left) and genus (Fig. 2A right) taxonomic level. After all count
189 data of taxonomy were converted to relative abundance, the top 10 abundant taxonomic groups
190 in each family and genus level were plotted in colored bar plot (43) (44) (45). Variation of
191 microbiome composition per DNA extraction method or per individual was assessed by the
192 Adonis test (`q2-diversity adonis`) (46) (47).

193

194 **Differentially accessible microbiota by DNA extraction protocol**

195 We set out to determine which microbial taxonomic groups were differentially accessible across
196 the sampling protocols by linear discriminant analysis (LDA) effect size (LEfSe) analyses (48).
197 We further assessed the microbial taxa using jvenn (49) at family and genus level. The Venn
198 diagram was created after removing OTUs with a frequency of less than 0.005% (42).

199

200 **Alpha and beta diversity analyses with or without rarefying**

201 Non-rarefying approaches to determine both alpha (within-sample) and beta (between-sample)
202 diversity was assessed by Species richness using `q2-breakaway` (50) and Aitchison distance
203 using `q2-deicode` (51). These were compared with rarefied data in which we applied Faith's
204 Phylogenetic Diversity, Observed OTUs, Shannon's diversity index, Pielou's Evenness,
205 Unweighted UniFrac distance, Weighted UniFrac distance, Jaccard distance, and Bray-Curtis
206 distances via `q2-diversity` (33). In order to retain data from at least 15 of the 20 patients (*i.e.*
207 75%; four samples from each of the four DNA extraction methods), we set the sampling depth to

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208 51,197 reads per sample. Overall our subsequence analysis consisted of 3,071,820 reads (27.6%,
209 3,071,820 / 11,149,582 reads). All diversity measurements in this study are listed in Table S1.
210

211 **Community type and HPV status**

212 In addition to the analysis above, we tested whether the samples clustered by microbiome
213 composition were related to the patient's clinical and demographic characteristics such as,
214 cervical biopsy diagnosis, race, and HPV16 status. HPV16 status has been reported to be
215 associated with both racial differences as well as microbial community types (26) (52) (53) (54).
216 We employed the Dirichlet Multinomial Mixtures (DMM) (55) model to determine the number
217 of community types for bacterial cervical microbiome. Then, we clustered samples to the
218 community type (9) (56). Since vaginal microbiota were reported to be clustered with different
219 *Lactobacillus sp*. such as *L. crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii* (16) (57), we also
220 collapsed the taxonomy to the species level and performed a clustering analysis using
221 "microbiome R package" (45). We then determined which bacterial taxa were differentially
222 abundant among the patients with or without HPV16 via `q2-aldex2` (58) and LEfSe (48).
223

224 **General statistical analysis**

225 All data are presented as means ± standard deviation (SD). Comparisons were conducted with
226 Fisher's exact test or Dunn's test with Benjamini-Hochberg-adjustment (59) or Wilcoxon test
227 with Benjamini-Hochberg-adjustment or pairwise PERMANOVA when appropriate. A p value <
228 0.05 or a q value < 0.05 was considered statistically significant.
229

230 **Data availability**

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231 MIMARKS compliant (60) DNA sequencing data are available via the Sequence Read Archive
232 (SRA) at the National Center for Biotechnology Information (NCBI), under the BioProject
233 Accession: PRJNA598197.

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234 **Results**

235 **Patients characteristics**

236 The age of the patients was 31.4 ± 5.0 years. The distribution of race was 15% African American
237 (n = 3) and 50% Caucasian (n = 10), and 35% Hispanic (n = 7). Cervical histology was 40%
238 CIN2 (n = 8), 50% CIN3 (n = 10), and 10% benign (n = 2). HPV genotype were 50% HPV16
239 positive (n = 10), 10% HPV18 positive (n = 2), 90% HR-HPV positives (n = 18). Patient
240 characteristics were summarized in Table 2.

241

242 **DNA yield**

243 DNA yield per 100 μL ThinPrep solution were 0.09 ± 0.06 μg in ZymoBIOMICS, 0.04 ± 0.01 μg
244 in PowerFecalPro, and 0.21 ± 0.23 μg in QIAampMini. DNA yield was not calculated for
245 IndiSpin, as Poly-A Carrier DNA was used. The DNA yield of PowerFecalPro was significantly
246 lower than that of ZymoBIOMICS (adjusted p value < 0.001) and QIAampMini (adjusted p
247 value < 0.001) based on Dunn's test with Benjamini-Hochberg-adjustment (Fig. S1).

248

249 **Number of reads and OTUs before rarefying**

250 We obtained a total of 11,149,582 reads for 80 DNA extractions. The 127,142 reads were
251 produced from a positive control of mock sample and 1,773 reads from ThinPrep solution as the
252 negative control. IndiSpin (168,349 ± 57,451 reads) produced a significantly higher number of
253 reads compared to PowerFecalPro (115,610 ± 68,201 reads, p value = 0.020, Dunn's test with
254 Benjamini-Hochberg-adjustment) as shown in Table 3. Approximately 90% of reads were
255 assigned to gram-positive bacteria and about 10% of reads were assigned to gram-negative
256 bacteria across all kits.

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257 Prior to rarefying, the ZymoBIOMICS kit captured a greater representation of gram-
258 negative bacterial OTUs (total 346, 17.3 ± 9.8) compared to PowerFecalPro (total 209, 10.5 ±
259 10.3, p value = 0.012, Dunn's test with Benjamini-Hochberg-adjustment, ratio of gram-negative
260 bacteria: 41.9% vs 33.7%) as shown in Table 3. No significant differences in the number of
261 OTUs before rarefying was detected for the entire bacterial community or gram-positive bacteria.
262

263 **Microbiome composition per DNA extraction protocol**

264 We analyzed whether differences in DNA extraction methods affect our ability to assess cervical
265 microbiota composition. The patients can be identified by whether or not they displayed a
266 *Lactobacillus*-dominant community type (Fig. 2A). Variation between individuals was a
267 significantly greater influence on the observed microbial composition than was the method of
268 DNA extraction (Fig. 2A).
269 The following top 10 abundant families are shown in Fig. 2A (left) and constituted
270 approximately 95.7% of cervical bacteria in all kits (80 DNA extractions); *Lactobacillaceae*
271 (58.9%), *Bifidobacteriaceae* (13.7%), *Veillonellaceae* (4.8%), *Prevotellaceae* (4.3%), *Family XI*
272 (3.9%), *Atopobiaceae* (3.0%), *Leptotrichiaceae* (2.5%), *Streptococcaceae* (2.0%),
273 *Lachnospiraceae* (1.6%). *Ruminococcaceae* (0.9%). The following top 10 abundant genera are
274 shown in Fig. 2A (right) and constituted approximately 92% of cervical bacteria; *Lactobacillus*
275 (58.9%), *Gardnerella* (13.6%), *Prevotella* (4.2%), *Megasphaera* (3.7%), *Atopobium* (3.0%),
276 *Sneathia* (2.5%), *Streptococcus* (1.9%), *Parvimonas* (1.7%), *Shuttleworthia* (1.4%), and
277 *Anaerococcus* (1.1%).
278

279 **Shared and unique microbiota among DNA extraction protocols**

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280 All DNA extraction methods were generally commensurate with one another, there were 31 of
281 41 shared microbes at the family level (Fig. 2B left) and 45 of 57 shared microbes at the genus
282 level (Fig. 2B right) among the DNA extraction protocols.
283 However, four gram-negative taxa were uniquely detected by ZymoBIOMICS and one
284 taxon was uniquely detected by QIAampMini both at the genus level (Fig. 2B right). Of the
285 uniquely detected ZymoBIOMICS OTUs, *Hydrogenophilus*, which was reported as enriched
286 taxa in LR-HPV positive environment (3), was detected in 14 of the 80 DNA extractions,
287 consisting of 2,488 reads (0.02% of all kit extractions). *Methylobacterium* was detected in 5 of
288 the 80 DNA extractions (912 reads; 0.01%). A member of this genus, *Methylobacterium*
289 *aerolatum*, has been reported to be more abundant in the endocervix than the vagina of healthy
290 South African women (61). *Bacteroidetes*, which are often reported as enriched taxa in an HIV
291 positive cervical environment (62), was detected in 12 of the 80 DNA extractions (1,028 reads;
292 0.01%). *Meiothermus* was detected in 9 of the 80 DNA extractions (882 reads; 0.01%).
293 *Meiothermus* is not considered to reside within the human environment, and may be an
294 extraction kit contaminant, as previously reported (63). A unique gram-positive taxa obtained
295 from the QIAampMini, *Streptomyces*, which was reported to be detected from the cervicovaginal
296 environment in the study of Kenyan women (64), was detected in 20 of 80 DNA extractions
297 (6,862 reads; 0.06%). No unique taxa were detected in PowerFecalPro and IndiSpin.
298 Venn diagrams at family levels also exhibited that ZymoBIOMICS detected slightly
299 more bacterial taxa (four unique taxa) as shown in Fig. 2B (left). These results showed that major
300 bacteria were commonly detected among all extraction protocols, with only slightly more
301 uniquely detected microbiota using ZymoBIOMICS.
302

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303 **Alpha and beta diversity**

304 Significantly higher Species richness (`q2-breakaway`) was observed from the
305 ZymoBIOMICS (56.1 ± 19.4) protocol compared to that of PowerFecalPro (43.2 ± 32.9, p =
306 0.025), QIAampMini (54.9 ± 29.8, not significant), and IndiSpin (63.6 ± 38.3, not significant)
307 using Dunn's test with Benjamini-Hochberg-adjustment (Fig. 3). Similarly, Faith's Phylogenetic
308 Diversity was observed to be higher with the ZymoBIOMICS protocol (6.6 ± 2.2), compared to
309 PowerFecalPro (4.5 ± 1.9, p = 0.012), QIAampMini (5.0 ± 1.8, not significant), and IndiSpin
310 (5.4 ± 1.7, not significant) using Dunn's test with Benjamini-Hochberg-adjustment (Fig. 3). The
311 use of IndiSpin also resulted significantly higher alpha diversity than that of PowerFecalPro in an
312 analysis of Species richness (p = 0.042, Dunn's test with Benjamini-Hochberg-adjustment). Non-
313 phylogenetic alpha diversity metrics such as Observed OTUs, Shannon's diversity index, and
314 Pielou's Evenness did not show differences among the four methods.

315 ZymoBIOMICS was able to significantly increase access to several taxonomic groups
316 compared to the other DNA extraction methods. Additionally, as shown in Table 4,
317 ZymoBIOMICS did capture a different microbial composition compared to other DNA
318 extraction methods in the index of Unweighted UniFrac distances (PowerFecalPro: q = 0.002;
319 QIAampMini: q = 0.002;and IndiSpin: q = 0.002) and in Jaccard distances (QIAampMini: q =
320 0.018 and IndiSpin: q = 0.033).

321

322 **Differential accessibility of microbiota by DNA extraction protocol**

323 LEfSe analysis identified taxonomic groups, defined with an LDA score of 2 or higher,
324 for differential accessibility by extraction kit: 23 in ZymoBIOMICS, 0 in PowerFecalPro, 3 in
325 QIAampMini, and 3 in IndiSpin (Fig. 4A). The following taxa were found to be highly

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326 accessible (LDA score > 3) with the use of the ZymoBIOMICS kit: Phylum *Proteobacteria*,
327 Class *Gammaproteobacteria*, Order *Betaproteobacteriales*, Family *Bacillaceae*, and Genus
328 *Anoxybacillus*. Whereas the Order *Streptomycetales* was highly enriched with the use of the
329 QIAampMini (LDA score > 3). As shown in the cladogram (Fig. 4B), despite the detection of a
330 potential kit contaminant (*Meiothermus sp.* a member of the Phylum *Deinococcus-Thermus*),
331 ZymoBIOMICS was able to increase the accessibility to additional microbiota compared to the
332 other extraction protocols.
333

334 **Microbial community type and HPV16**

335 DMM detected two cervical microbial community types across all four DNA extraction
336 protocols. Community type I was composed of the following: *Gardnerella sp.* (ZymoBIOMICS:
337 17.1%; PowerFecalPro: 20%; QIAampMini: 23%; IndiSpin: 20%), *Lactobacillus iners*
338 (ZymoBIOMICS: 6.3%; PowerFecalPro: 5%; QIAampMini: 6%; IndiSpin: 5%), *Atopobium*
339 *vaginae* (10) (ZymoBIOMICS: 3.5%; PowerFecalPro: 3%; QIAampMini: 4%; IndiSpin: 5%),
340 *Clamydia trachomatis* (ZymoBIOMICS: 1.9%; PowerFecalPro: 2%; QIAampMini: 3%;
341 IndiSpin: 2%), *Shuttleworthia sp.* (ZymoBIOMICS: 1.8%; PowerFecalPro: 2%; QIAampMini:
342 2%; IndiSpin: 2%). Some members of *Shuttleworthia* are considered to be bacterial
343 vaginosis□associated bacterium (BVAB) (65), further investigation is required to determine if
344 this OTU is indeed a BVAB. We determined this community type "high diversity type".
345 Community type II was is dominated by *Lactobacillus iners* at 88%, 85%, 83%, and 85%
346 respectively for ZymoBIOMICS, PowerFecalPro, QIAampMini, and IndiSpin.
347 The relationship between HPV16 infection and community type was observed to be
348 significantly associated with community type I (HPV16 positive patients [n = 9], HPV16

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349 negative patients [n = 1]) and not community type II (HPV16 positive patients [n = 1], HPV16
350 negative patients [n = 9], p = 0.001, Fisher's exact test) regardless of the DNA extraction kit used.
351 In support of this result, analysis of differentially abundant microbiota using `q2-aldex`
352 (Benjamini-Hochberg corrected p value of Wilcoxon test: p < 0.001, standardized distributional
353 effect size: –1.2) revealed that *Lactobacillus iners* were differentially enriched in the cervical
354 environment without HPV16. LEfSe analysis also detected that genus *Lactobacillus* were
355 enriched in the cervical environment without HPV16 (p < 0.001, LDA score: –5.38). No
356 significant differences were observed in the relationship between community type and HPV18 (p
357 = 0.474, Fisher's exact test), HR-HPV (p = 0.474, Fisher's exact test), results of cervical biopsy
358 (p = 0.554, Fisher's exact test), and race (African Americans vs not-African Americans: p = 1;
359 Caucasian vs not-Caucasian: p = 0.656; Hispanic vs not-Hispanic: p = 0.350, Fisher's exact test).

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360 **Discussion**

361 In this study, we evaluated the utility of LBC specimens for the collection and storage of cervical
362 samples for microbiome surveys based on the 16S rRNA marker gene. We simultaneously
363 compared the efficacy of several commonly used DNA extraction protocols on these samples in
364 an effort to develop a standard operating procedure/protocol (SOP) for such work. We've also
365 been able to show that there are two cervical microbial community types, which are associated
366 with the dominance or non-dominance of *Lactobacillis iners*. Both community types were
367 detected regardless of the DNA extraction protocol used.

368 This study evaluated the composition of microbiota across all DNA extraction methods.
369 These findings document the importance of selecting DNA extraction methods in cervical
370 microbiome studies from the LBC samples. All kits were commensurate in their ability to
371 capture the microbial composition of each patient and the two observed cervical microbial
372 community state types: making all of these protocols viable for discovering broad patterns of
373 microbial diversity. However, we did observe that the ZymoBIOMICS protocol was better able
374 to access additional cervical microbiota (Fig. 2B, 4A & B). Coincidentally, we detected potential
375 DNA contamination only with the ZymoBIOMICS kit. The number of OTUs prior to rarefying
376 revealed that the ZymoBIOMICS protocol detected more gram-negative OTUs than the
377 PowerFecalPro (Table 3 & Fig. 2B). In particular, LEfSe analysis has shown that phylum
378 *Proteobacteria* can be better detected with the ZymoBIOMICS kit (Fig. 4). Although rarefying
379 microbiome data can be problematic (66), it can still provide robust and interpretable results for
380 diversity analysis (67), we were able to observe commensurate findings with non-rarefying
381 approaches such as `q2-breakaway` (50), `q2-deicode` (51), and LEfSe (48). Beta-diversity
382 analysis via Unweighted UniFrac also revealed that ZymoBIOMICS was significantly different

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383 from all other kits. There were no differences in non-phylogenic indices of alpha diversity with
384 rarefying approaches. These findings lead us to surmise that phylogenetic indices may be more
385 sensitive than the non-phylogenetic indices.

386 Although we hypothesized that the detection of difficult-to-lyse-bacteria (*e.g*. gram-
387 positive bacteria) would vary by kit, we observed no significant differences (Table 3). As shown
388 in Table 3, the number of reads of gram-positive and gram-negative bacteria also showed that
389 there was no difference in the four kits. This is likely due to several modifications made to the
390 extraction protocol as outlined in Table 1. That is, we added bead beating and mutanolysin to the
391 QIAampMini protocol (68). We also modified the beating time of the ZymoBIOMICS kit down
392 to 2 minutes from 10 minutes (the latter being recommended by the manufacturer) to minimize
393 DNA shearing. We may use the extracted DNA from ZymoBIOMICS for long-read amplicon
394 sequencing platforms such as PacBio (Pacific Biosciences of California, Inc) (69) or MinION
395 (Oxford Nanopore Technologies) (70) (71). Excessive shearing can render these samples
396 unusable for long-read sequencing. It is quite possible that we could have observed even more
397 diversity with the ZymoBIOMICS kit for our amplicon survey if we conducted bead-beating for
398 the full 10 minutes.

399 Community typing and detection of the differentially abundant microbiota revealed that
400 *Lactobacillus iners* were more abundant in the cervical ecosystem without HPV16. These
401 findings are congruent with those of Lee *et al*. (1) and Audirac-Chalifour *et al*. (72). Lee *et al*.
402 reported that *Lactobacillus iners* were decreased in women with HPV positive (1). Also, the
403 result that the proportion of *Lactobacillus iners* was higher in HPV-negative women compared to
404 HPV-positive women (relative abundance 14.9% vs 2.1%) was reported by Audirac-Chalifour *et*
405 *al* (72). Similarly, Tuominen *et al*. (18) reported that *Lactobacillus iners* were enriched in HPV

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406 negative samples (relative abundance: 47.7%) compared to HPV positive samples (relative
407 abundance: 18.6%, p value = 0.07) in the study of HPV positive-pregnant women (HPV16
408 positive rate: 15%). As established by the seminal study of Ranjeva *et al*. (73), a statistical model
409 revealed that colonization of specific HPV type including multi HPV type infection depends on
410 host-risk factors such as sexual behavior, race and ethnicity, and smoking. It is unclear whether
411 the association between the cervical microbiome, host-specific traits, persistent infection of
412 specific HPV types, such as HPV16, can be generalized and requires further investigation.

413 We focused on LBC samples as this is the recommended method of storage for cervical
414 cytology (74). Here, we confirmed that LBC samples can be used for microbial community
415 surveys by simply using the remaining LBC solution post HPV testing or cervical cytology. We
416 used a sample volume of 200 or 300 μL ThinPrep solution in this study. HPV genotyping test
417 using Linear Array HPV Genotyping Test (Roche Diagnostics) stably detects β-globin with a
418 base length of 268 bp as a positive control. Therefore, using a similar sample volume as HPV
419 genotyping (250 μL), it was expected that V4 (250 bp), which is near the base length of β-globin,
420 would be PCR amplified. It has been pointed out by Ling *et al*. (75) that the cervical
421 environment is of low microbial biomass. To control reagent DNA contamination and estimate
422 the sample volume, DNA quantification by qPCR before sequencing is recommended (76). Mitra
423 *et al* determined a sample volume of 500 μL for ThinPrep by qPCR in the microbiome study
424 comparing sampling methods using cytobrush or swab from cervix (19). The average storage
425 period from sample collection via LBC to DNA extraction was about two years in this study.
426 Kim *et al*. reported that DNA from cervix stored in ThinPrep at room temperature or −80°C was
427 stable for at least one year (77). Meanwhile, Castle *et al*. reported that β-globin DNA fragments
428 of 268 bases or more were detected by PCR in 90 % (27 of 30 samples) of ThinPrep samples

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429 stored for eight years at an uncontrolled ambient temperatures followed by a controlled ambient
430 environment (10–26.7°C) (78). Low-temperature storage may allow the analysis of the short
431 DNA fragments of the V4 region after even long-term storage, although further research is
432 needed to confirm the optimal storage period in cervical microbiome studies using ThinPrep.
433 SurePath LBC specimens are as widely used as ThinPrep, but the presence of formaldehyde
434 within the SurePath preservation solution raises concerns about accessing enough DNA for
435 analysis as compared to ThinPrep, which contains methanol (79) (80). It should also be noted
436 that other storage solutions, *i.e*. those using guanidine thiocyanate have been reported for
437 microbiome surveys of the cervix (81) and feces (82). A weakness of the current study is that we
438 did not examine the reproducibility of our results as each sample was extracted using each kit
439 once. However, the use of actual patient samples rather than mock samples is a strength of our
440 approach.

441 In conclusion, regardless of the extraction protocol used, all kits provided equivalent
442 accessibility to the cervical microbiome. All kits shared the ability to access 31 of 41 families
443 and 45 of 57 genera (Fig. 2), approximately 90% of bacteria were gram-positive and 10% were
444 gram-negative. Observed differences in microbial composition were due to the significant
445 influence of the individual patient and not the extraction protocol. However, ZymoBIOMICS
446 was observed to increase the accessibility of DNA from a greater range of microbiota compared
447 to the other kits, in that the greatest number of significantly enriched taxa were identified (Fig. 3).
448 This was not because of higher DNA yield nor ability to detect more gram-positive bacteria.
449 Selection and characterization of an appropriate DNA extraction methods, for providing accurate
450 census of cervical microbiota, and the human microbiome in general are important (23) (24) (25)
451 (68) (77) (78). We have shown that the ability to characterize cervical microbiota from LBC

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452 specimens is robust, even after prolonged storage. Our data also suggest that it is possible to
453 reliably assess the relationship between HPV and the cervical microbiome, also supported by
454 Kim *et al*. (77) and Castle *et al* (78). Even though we found all four extraction kits to be
455 commensurate in their ability to broadly characterize the CM, this study lends support to the
456 view that the selection of a DNA extraction kit depends on the questions asked of the data, and
457 should be taken into account for any cervicovaginal microbiome and HPV research that
458 leverages LBC specimens for use in clinical practice (15) (83).
459

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468 analysis and wrote paper. T.S., H.C., and M.N. created the protocol of DNA extraction. M.N.,
469 H.C., S.O., W.G., and T.S. provided important feedback. Samples in the clinical trial were
470 collected by W.G. and his associates. DNA extraction was conducted by T.S. Sequencing of 16S
471 RNA gene was conducted by S.O.

472 M.N. is one of the inventors named in the patents and patent applications for the HPV
473 therapeutic vaccine PepCan. The remaining authors declare no conflicts of interest.

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Table 1: Characteristics of four different DNA extraction protocols

Kit (Cat. No.)	Manufacturer	Sample volume	Enzyme	Beads	Beating	DNA carrier	Others
ZymoBIOMICS DNA Miniprep Kit (D4300)	Zymo Research	300 µL	No	Ceramic[a]	2 min[b]	No	[c]
QIAamp PowerFecal Pro DNA Kit (51804)	Qiagen	300 µL	No	Ceramic[d]	10 min[b]	No	[c]
QIAamp DNA Mini Kit (51304)	Qiagen	200 µL	Mutanolysin[e]	No	No	No	[c, f, g]
IndiSpin Pathogen Kit (SPS4104)	Indical Bioscience	300 µL	No	Ceramic[h]	10 min[b]	Yes	[c, i]

a: https://www.zymoresearch.com/pages/microbial-isolation. b: Disruptor Genie (USA Scientific, Inc.) was used under the maximum speed. c: Nuclease free water (85 µL) as DNA elution buffer was used. d: PowerBead Pro Tubes (https://www.qiagen.com/us/products/discovery-and-translational-research/lab-essentials/plastics/powerbead-tubes/#orderinginformation). e: Instead of lysozyme or lysostaphin, mutanolysin was used as per Yuan *et al*, 2012 (68). f: DNA Purification from Blood or Body Fluids; Protocols for Bacteria; Isolation of genomic DNA from gram-positive bacteria in QIAamp DNA Mini and Blood Mini Handbook fifth edition was referenced. g: Heating at 56°C for 30 min and 95°C for 15 min was performed. h: Pathogen Lysis Tubes S (https://www.qiagen.com/dk/shop/pcr/pathogen-lysis-tubes/). i: Pretreatment B2 as per QIAamp cador Pathogen Mini Handbook.

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Table 2. Patients characteristics

Characteristics		Values
Number of patients, n		20
Total number of DNA extracts, n		80
Age, mean (SD)		31.4 (5.0)
Race	African American, n (%)	3 (15)
	Caucasian, n (%)	10 (50)
	Hispanic, n (%)	7 (35)
Cervical biopsy	CIN2, n (%)	8 (40)
	CIN3, n (%)	10 (50)
	Benign, n (%)	2 (10)
HPV typing	HPV positive, n (%)	19 (95)
	HPV16 positive, n (%)	10 (50)
	HPV18 positive, n (%)	2 (10)
	HPV16 or 18 positives, n (%)	10 (50)
	HR-HPV positives, n (%)	18 (90)

SD: standard deviation. CIN: cervical intraepithelial neoplasia. HR-HPV: high-risk HPV (HPV16 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68)

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Table 3. Reads and OTUs before rarefying assigned to all, gram-, and gram-negative bacteria per DNA extraction protocols

Parameters	Community	Methods	Values	Ratio of GP or GN	p value
Number of reads (mean ± SD)	All	Zy	2,705,044 (135,252 ± 66,011)		*a*
		Pro	2,312,207 (115,610 ± 68,201)		
		QIA	2,765,343 (138,267 ± 49,781)		
		IN	3,366,988 (168,349 ± 57,451)		
	GP	Zy	2,430,380 (121,519 ± 56,209)	89.8%	NS
		Pro	2,116,458 (105,823 ± 57,590)	91.5%	
		QIA	2,503,578 (125,179 ± 46,073)	90.5%	
		IN	2,985,941 (149,297 ± 46,936)	88.7%	
	GN	Zy	274,664 (13,733 ± 29,162)	10.2%	NS
		Pro	195,749 (9,788 ± 23,070)	8.5%	
		QIA	261,765 (13,088 ± 22,638)	9.5%	
		IN	381,047 (19,052 ± 33,038)	11.3%	
Number of OTUs (mean ± SD)	All	Zy	825 (41.3 ± 16.8)		NS
		Pro	621 (31.1 ± 19.4)		
		QIA	778 (38.9 ± 22.4)		
		IN	792 (39.6 ± 22.7)		
	GP	Zy	479 (24.0 ± 9.2)	58.1%	NS
		Pro	412 (20.6 ± 12.7)	66.3%	
		QIA	513 (25.7 ± 13.7)	65.9%	
		IN	531 (26.6 ± 14.9)	67.0%	
	GN	Zy	346 (17.3 ± 9.8)	41.9%	*b*
		Pro	209 (10.5 ± 10.3)	33.7%	
		QIA	265 (13.3 ± 9.2)	34.1%	
		IN	261 (13.1 ± 8.3)	33.0%	

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Community of gram-positive bacteria were defined as phylum *Actinobacteria* and *Firmicutes*, which are composed of thick peptidoglycan layers without outer membrane (84). Community of gram-negative bacteria was defined as a community of bacteria other than phylum *Actinobacteria* and *Firmicutes* in this study. a: I - P: 0.0199; I - Q: 0.1590; P - Q: 0.1436; I - Z: 0.1495; P - Z: 0.1712; and Q - Z: 0.4059. b: I - P: 0.2116; I - Q: 0.4837; P - Q: 0.1143; I - Z: 0.0938; P - Z: 0.0116; Q - Z: 0.1448. Dunn's test with Benjamini-Hochberg-adjustment were performed for comparison of the number of read and OTU by DNA extraction method. Zy: ZymoBIOMICS DNA Miniprep Kit, Pro: QIAamp PowerFecal Pro DNA Kit, QIA: QIAamp DNA Mini Kit, IN: IndiSpin Pathogen Kit. SD: standard deviation. All: all bacteria, GP: gram-positive bacteria, GN: gram-negative bacteria. NS: not significant.

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Table 4. Beta diversity among DNA extraction methods

Index	Protocol	Protocols compared	p values	q values
Aitchison distance	ZymoBIOMICS	PowerFecalPro	NS	NS
		QIAampMini	NS	NS
		IndiSpin	NS	NS
	PowerFecalPro	QIAampMini	NS	NS
		IndiSpin	NS	NS
	QIAampMini	IndiSpin	NS	NS
Unweighted UniFrac distance	ZymoBIOMICS	PowerFecalPro	0.001	0.002
		QIAampMini	0.001	0.002
		IndiSpin	0.001	0.002
	PowerFecalPro	QIAampMini	NS	NS
		IndiSpin	0.015	0.023
	QIAampMini	IndiSpin	NS	NS
Weighted UniFrac distance	ZymoBIOMICS	PowerFecalPro	NS	NS
		QIAampMini	NS	NS
		IndiSpin	NS	NS
	PowerFecalPro	QIAampMini	NS	NS
		IndiSpin	NS	NS
	QIAampMini	IndiSpin	NS	NS
Jaccard distance	ZymoBIOMICS	PowerFecalPro	0.037	NS
		QIAampMini	0.003	0.018
		IndiSpin	0.011	0.033
	PowerFecalPro	QIAampMini	NS	NS
		IndiSpin	NS	NS
	QIAampMini	IndiSpin	NS	NS
Bray-Curtis distance	ZymoBIOMICS	PowerFecalPro	NS	NS

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	QIAampMini	NS	NS
	IndiSpin	NS	NS
PowerFecalPro	QIAampMini	NS	NS
	IndiSpin	NS	NS
QIAampMini	IndiSpin	NS	NS

Pairwise PERMANOVA was tested for comparing beta diversity of DNA extraction method. NS: not significant.

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Table S1. Diversity analysis in this study

No.	Parameter	Alpha or Beta diversity	Used data with/without rarefying	Input data with/without phylogenetic information	Plugin of QIIME 2
1	Species richness	Alpha	Not rarefied	Non-phylogenetic	`q2-breakaway` (50)
2	Faith's Phylogenetic Diversity	Alpha	Rarefied	Phylogenetic	`q2-diversity`
3	Observed OTUs	Alpha	Rarefied	Non-phylogenetic	`q2-diversity`
4	Shannon's diversity index	Alpha	Rarefied	Non-phylogenetic	`q2-diversity`
5	Pielou's Evenness	Alpha	Rarefied	Non-phylogenetic	`q2-diversity`
6	Aitchison distance	Beta	Not rarefied	Non-phylogenetic	`q2-deicode` (51)
7	Unweighted UniFrac distance	Beta	Rarefied	Phylogenetic	`q2-diversity`
8	Weighted UniFrac distance	Beta	Rarefied	Phylogenetic	`q2-diversity`
9	Jaccard distance	Beta	Rarefied	Non-phylogenetic	`q2-diversity`
10	Bray-Curtis distances	Beta	Rarefied	Non-phylogenetic	`q2-diversity`
11	Adonis	Beta	Rarefied	Non-phylogenetic	`q2-diversity adonis` (46) (47)

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756 **Figure legends**

757 **Fig. 1. Overview of the study design using the 16S rRNA gene to compare the DNA**
758 **extraction protocol for cervical microbiota.** (A) Liquid-based cytology (LBC) specimens from
759 20 patients with CIN2/3 or suspected CIN2/3. (B) A total of 80 DNA extractions were performed.
760 (C) The four DNA extraction methods. (D) DNA of mock vaginal community as a positive
761 control and preservation solution as a negative control. (E) Sequencing using Illumina MiSeq.
762 (F) Analysis of the taxonomic profiles among the DNA extraction protocols. Images form Togo
763 Picture Gallery (http://togotv.dbcls.jp/ja/pics.html) were used to create this figure.
764

765 **Fig. 2. Taxonomic resolution among DNA extraction protocols.** (A) Relative abundance of
766 microbe at family level (left) and genus level (right) per DNA extraction method showed the
767 pattern that variance of microbe composition per patient was higher than that per DNA extraction
768 protocol. These pattern were confirmed by values of Adonis test (`q2-diversity adonis`);
769 F.Model: 199.4, R2: 0.982, and p value: 0.001 for patients and F.Model: 2.9, R2: 0.003, and p
770 value: 0.002 for DNA extraction (46) (47). After all count data of taxonomy were converted to
771 relative abundance as shown in the y-axis, the top ten taxonomy at each family and genus level
772 were plotted in colored bar plot and other relatively few taxonomies were not plotted. The 20
773 patients ID were described in the x-axis. (B) Venn diagrams showed that ZymoBIOMICS had
774 four unique taxa at family (left) and genus (right) taxonomic level. Thirty-one of 41 families and
775 45 of 57 genera were detected with all DNA extraction protocols.
776

777 **Fig. 3. Comparisons of alpha diversity between different DNA extraction protocols.** The
778 alpha diversity indices determined by Species richness and Phylogenetic diversity are

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779 significantly higher with ZymoBIOMICS in comparison with PowerFecalPro (p = 0.025 and
780 0.012, respectively, Dunn's test with Benjamini-Hochberg-adjustment). IndiSpin also showed
781 significantly higher diversity than that of PowerFecalPro using analysis of Species richness (p =
782 0.042, Dunn's test with Benjamini-Hochberg-adjustment). No significant differences were
783 observed in other alpha diversity indexes such as observed OTUs, Shannon's diversity index,
784 and Pielou's Evenness. Zy: ZymoBIOMICS DNA Miniprep Kit, Pro: QIAamp PowerFecal Pro
785 DNA Kit, QIA: QIAamp DNA Mini Kit, IN: IndiSpin Pathogen Kit.
786
787 **Fig. 4. Distinct detections of microbe among the DNA extraction protocols.** (A) A bar graph
788 showing 23 significantly enriched taxa with ZymoBIOMICS, 3 with QIAamp DNA Mini Kit,
789 and 3 with IndiSpin Pathogen Kit determined by the linear discriminant analysis (LDA) effect
790 size (LEfSe) analyses (48). (B) A taxonomic cladogram from the same LEfSe analyses showing
791 that the significantly enriched microbiota in ZymoBIOMICS were composed of phylum
792 *Proteobacteria*. Also note that *Meiothermus (a member of* the phylum *Deinococcus-Thermus)* is
793 likely an extraction kit contaminant. Zy: ZymoBIOMICS DNA Miniprep Kit, Pro: QIAamp
794 PowerFecal Pro DNA Kit, QIA: QIAamp DNA Mini Kit, IN: IndiSpin Pathogen Kit. g_: genus,
795 f_: family, o_: order, c_: class, p_: phylum.
796
797 **Fig. S1. Comparison of DNA yields by DNA extraction protocols.** DNA yield of
798 QIAampMini was significantly higher than that of PowerFecalPro ($p < 0.001$, Dunn's test with
799 Benjamini-Hochberg-adjustment). Also, the DNA yield of ZymoBIOMICS was significantly
800 higher than that of PowerFecalPro ($p < 0.001$, Dunn's test with Benjamini-Hochberg-adjustment).
801 The amount of DNA was calculated based on the absorbance of nucleic acids measured by

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802 Nanodrop One. By the protocol recommended by the manufacturer, nucleic acid (Poly-A carrier)
803 was used in IndiSpin. Therefore, IndiSpin was excluded from the analysis of DNA yield. The
804 amount of DNA yield per 100 μL ThinPrep sample volume were compared. The bar graph shows
805 the mean and standard deviation. Zy: ZymoBIOMICS DNA Miniprep Kit, Pro: QIAamp
806 PowerFecal Pro DNA Kit, QIA: QIAamp DNA Mini Kit.

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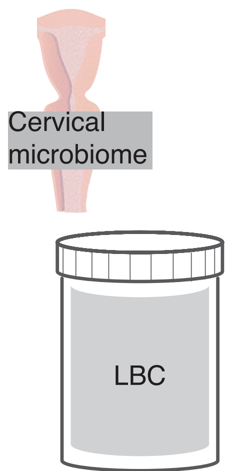
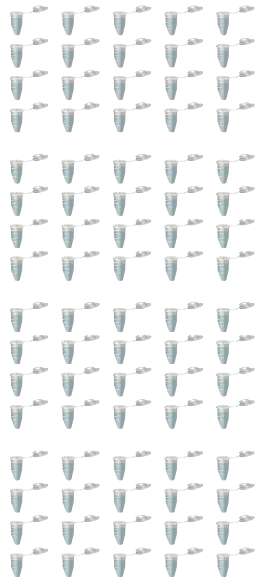
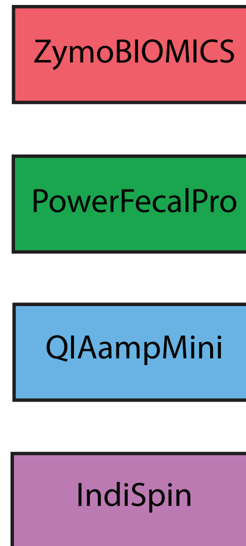
(A) Liquid-based cytology on cervix



(B) Dispensing of each sample to 4 aliquot



(C) DNA extraction using four protocols



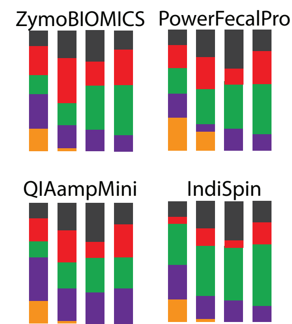
(D)

Positive control (vaginal mock)

Negative control (preservation solution without samples)

(E) 16S rRNA Illumina amplicon sequencing

(F) Evaluation of taxonomic profiles



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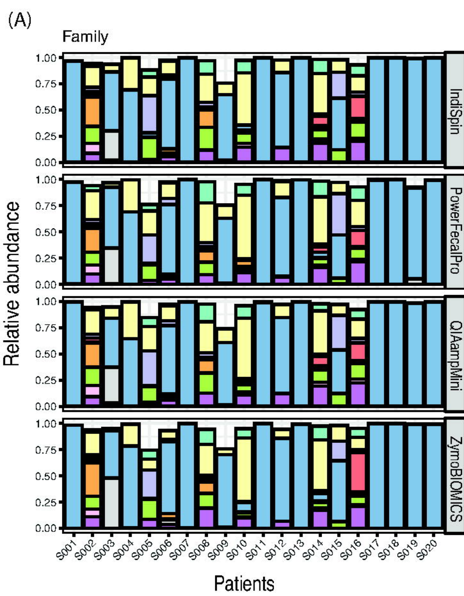
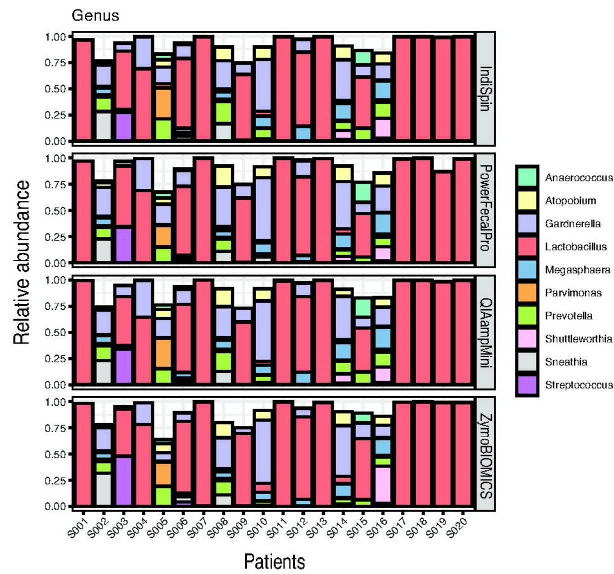
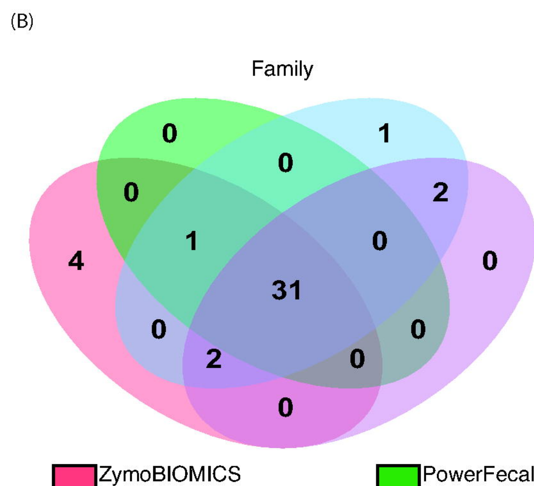
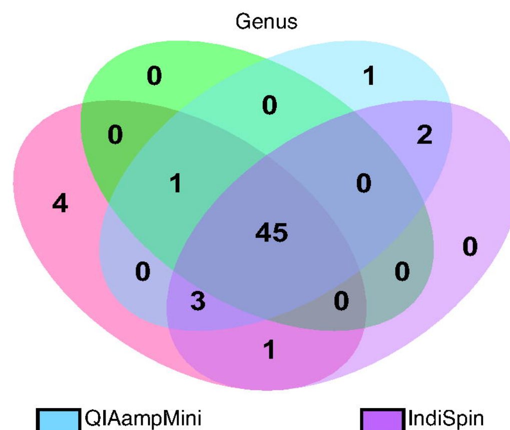
(A)





(B)





ZymoBIOMICS PowerFecalPro QIAampMini IndiSpin

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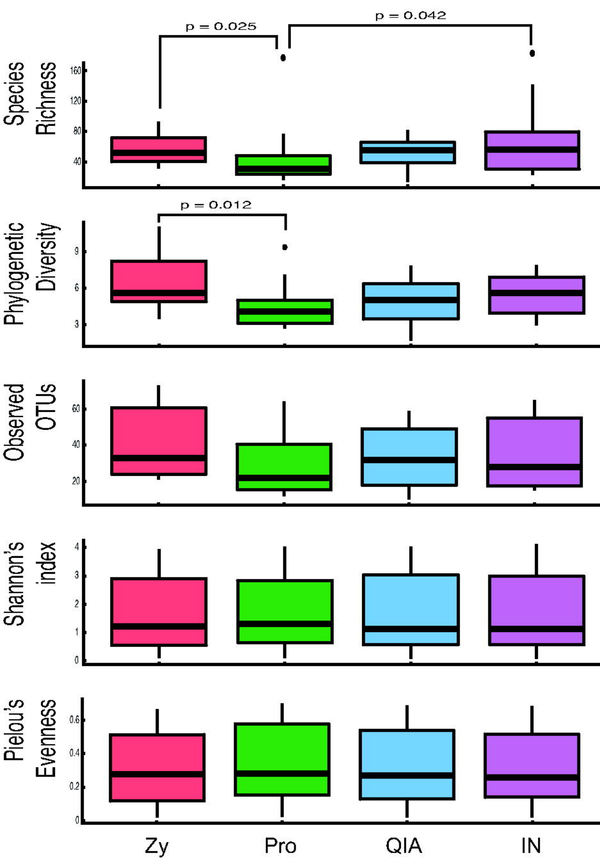

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