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1 **The influence of sex on the urogenital microbiome of rhesus monkeys**

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14 ***Abstract:***

15 The vaginal microbiome of nonhuman primates (NHPs) differs substantially from humans
16 in terms of *Lactobacillus* abundance, overall taxonomic diversity, and vaginal pH. Given
17 these differences, it remains unclear in what way the NHP genital microbiome protects
18 against pathogens, in particular sexually transmitted diseases. Considering the effect that
19 microbiome variations can have on disease acquisition and outcome, we examined
20 endogenous and exogenous factors that influence the urogenital microbiome of captive
21 rhesus monkeys. The male urethral (n=37) and vaginal (n=194) microbiome of 11
22 breeding groups were examined in a cross-sectional study. During lactation and
23 menstruation, the vaginal microbiome becomes significantly more diverse and more
24 similar to the microbes observed in the male urethra. Group association and cage-mate
25 (sexual partners) relationships were additionally associated with significant differences in
26 the urogenital microbiome. Our results demonstrate that microbiome considerations are
27 necessary in order to make informed selection of NHPs as translational animal models.

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28 **Introduction**

29 In recent years there has been an increased interest in the microbiome of nonhuman primates
30 (NHPs) for evolutionary, experimental, and conservation purposes. However, microbiome
31 considerations are currently not used to refine and reduce experiments with NHPs, despite
32 increasing evidence that the microbiome in humans can influence disease progression
33 (reviewed by (*1*)). Of the NHPs animal models, the Asian rhesus monkey (*Macaca mulatta*)
34 and long-tailed macaque (*Macaca fascicularis*) are the most extensively utilized species *(2-4)*.
35 In laboratory settings, rhesus monkeys cycle year-round, have a reproductive cycle that is
36 similar to that of humans and experience similar changes in the hormonal levels during sexual
37 cycle, pregnancy and post-partum (*5-7*). Therefore, the vagina of rhesus monkeys has been
38 used to model the human vaginal epithelium and study sexual transmitted infections (STIs)
39 (*8*). For example, rhesus monkeys have been extensively used to study the disease acquisition
40 and outcome of simian-/human immunodeficiency virus (SIV/HIV) (*3, 9*). In a study on SIV
41 susceptibility, estrogen treatment in rhesus monkeys protected female rhesus monkeys from
42 the sexually transmitted infection (*3*). *Smith et al*. propose that not just the thickening of the
43 vaginal epithelium but also a potential change in vaginal microenvironment may have led to
44 the observed effect under the influence of high estrogen levels (*3*).

45 Many studies have laid the groundwork in characterizing the genital
46 microenvironment of various species of captive and wild NHPs (2, *10-12*). Unlike the vaginal
47 microbiome of humans, which is often dominated by a single *Lactobacillus* species (*13*),
48 NHPs, including rhesus monkeys, harbor a diverse set of vaginal microbes (*2, 11*). In humans,
49 the acidic nature of the vaginal flora (pH≤4.5) protects women against STIs (*14*). The vaginal
50 microbiome of NHPs on the other hand, has a low abundance of *Lactobacillus* (<2% of
51 microbiome), an overall higher taxonomic diversity, and a near neutral vaginal pH (*2, 11, 12,*
52 *15*). Considering these differences, it currently remains unclear in what way the vaginal
53 microenvironment of rhesus monkeys protects against infectious diseases. Additionally,
54 despite increasing evidence that sexual exposures can alter the composition of the human
55 genital flora (*16, 17*), the urethral microbiome of male NHPs remains largely uncharacterized.
56 A better understanding of factors that influence the rhesus monkey genital microbiome of
57 both male and female animals in health and disease is thus warranted.

58 In this study, we investigate the genital microbiome of a large breeding colony of
59 rhesus monkeys at the German Primate Center. To identify endogenous and exogenous
60 factors that influence the microbiome, we examine the genital microbiome in the breeding
61 colonies in the context of age, breeding group association, social rank, body mass, and long-

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62 term health status. We studied both, the vaginal microbiome of female and the urethral
63 microbiome of male rhesus monkeys which has not been done in previous studies. We are
64 therefore able to compare bacterial composition between male and female animals in a single
65 cohort of rhesus monkeys and found that during menstruation and lactations the vaginal
66 microbiome shifts towards the male urethral microbiome.
67
68 **Results**
69 We examined the vaginal microbiome of 194 female rhesus monkeys and the urethral
70 microbiome of 37 male rhesus monkeys housed at the breeding facility of the German
71 Primate Center (data file S1). The mean age, number of breeding groups, and other
72 characteristics of the sampled animals are shown in Table 1. The V4 region of the bacterial
73 16S rRNA gene was amplified and sequenced on the Illumina MiSeq platform, generating a
74 total of 14,571,505 unfiltered reads with a mean read count of 48,630 reads per sample
75 (±15,461 SD) after quality filtering. Sequences were rarefying to 11,371 sequences per
76 sample and clustered into Operational taxonomic units (OTUs) based on the 97% similarity
77 threshold. We first examined the microbiome of the male urethra and vagina separately and
78 then compared composition similarities between male and female animals.

79

80 **Table 1: Characteristics of sampled rhesus monkeys in this study.**

	Female (n=194)	Male (n=37)
Mean age, years	10.0 ± 4.9	7.9 ± 5.6
n Geriatric (>19 years)	10	1
n Adult (5-19 years)	168	17
n Juvenile (< 5 years)	16	19
Breeding groups	11	10
Vaginal pH, mean ± SD	6.4 ± 0.7 (n=138)	N/A
% EVC*	76.3% (n=148)	N/A
n Phase 1	44†	N/A
n Phase 2	57†	N/A
n Phase 3	47†	N/A
% Lactating	30.4 (n=59)	N/A
% Dominant male	N/A	40.5 (n=15)

81
82 *Cytology phases classification described in Detail in Methods and Supplementary Table S2;
83 †For subdivision of sexual cycle phases by lactating status see Table S2
84

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85 Appropriate control samples and a mock community (Microbial mock community, HM-280,
86 Biodefense and Emerging Infectious Research (BEI) Resources, Manassas, USA) were
87 included in the sequencing run. Using the mock community, the observed error rate for the
88 run was found to be 0.036%. The collected control samples showed that contamination was
89 highest during sample collection procedures, while controls taken during amplification
90 procedures in the laboratory yielded only minimal read counts (Fig. S1A). Taxa plots of
91 control samples that were taken during sampling at two different breeding units, show that the
92 relative abundance of contaminant OTUs was similar between the units (Fig. S1B).

93 ***The vaginal microbiome is significantly altered during lactation and menstruation***

94 We investigated the vaginal microbiome (n=194), vaginal pH (n=138), and sexual cycle phase
95 (n=148) of clinically-healthy, reproductively-active rhesus monkeys housed in eleven
96 breeding groups (Table 1). None of the females showed signs of pregnancy, as defined by
97 transabdominal palpation. At the time of sampling 30.4% of the animals were lactating. In
98 addition to lactation, we characterized the sexual cycle phase using exfoliative vaginal
99 cytology (EVC) (see Methods, Table S1). The sexual cycle phase (P1-P3) of non-lactating
100 females were evenly distributed with 35.6% in an ovulatory phase (P1), 41.3% in an
101 intermediate phase (P2) and 23.1% in a menstruation-like phase (P3) (Table S1). For lactating
102 females, 52.3% of the animals were in a menstruation-like phase, 31.8% in an intermediate
103 phase, and 15.9% in the ovulatory phase (Table S1). For the purpose of the microbiome
104 analysis, lactation status and sexual cycle phases were analyzed independently.

105 Overall, a mean of 219.8±160.7 (unless otherwise stated all values are given in mean
106 ± SD) OTUs were observed in the vaginal microbiota of the rhesus monkeys. The most
107 abundant genus was *Prevotella* with a mean abundance of 20.5±16.4%. Different OTUs were
108 identified as *Prevotella*, indicating that a diverse set of species from this genus were present
109 (Fig. 1). *Porphyromonas* (9.5±9.9%), *Streptobacillus* (9.1±13.4%) and an unclassified genus
110 of the family *Ruminococcaceae* (9.5±7.3) were the other dominant taxa in the otherwise
111 diverse community (Fig. 1).
112

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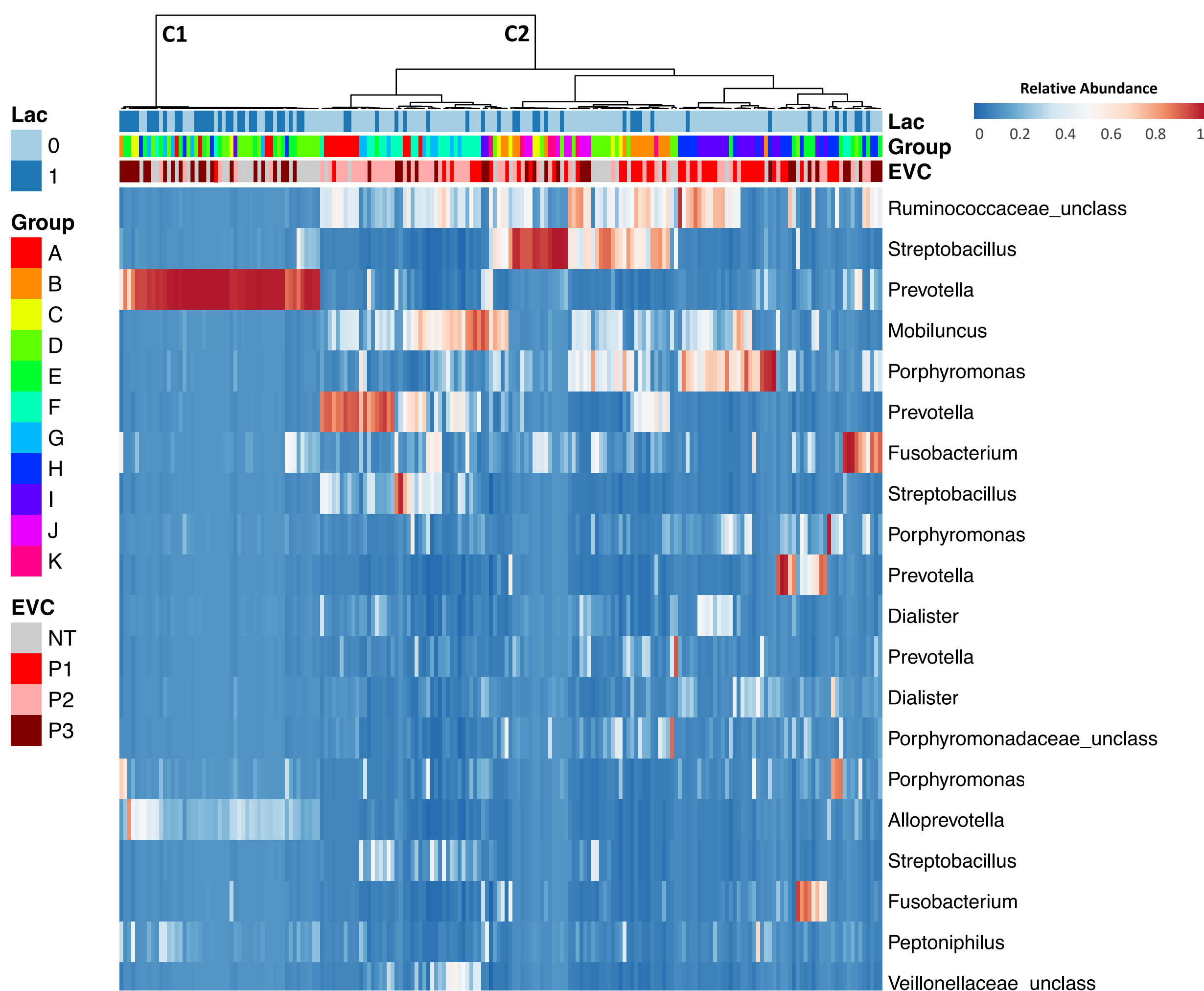

113
114
115 **Fig. 1: Heatmap of the relative abundance of microbial taxa identified in the vaginal**
116 **microbiota of rhesus monkeys in multiple breeding groups.** Ward linkage clustering of
117 samples based on the composition and relative abundance of the 20 most abundant OTUs in
118 the vaginal microbiota. Genus-level bacterial classification of OTUs shown with the percent
119 of sequences that classified with the specific genus. Lactation status (1: lactating and 2: non-
120 lactating), group association (A-K) and EVC (sexual cycle phases) (P1: ovulatory phase, P2:
121 intermediate stage, P3: menstruation-like and NT: not tested) of each sample are shown
122 beside the heatmap. C1 and C2 indicate two main clusters in the ward linkage clustering. See
123 Table 1 for sample size composition.
124
125 Lactation status and sexual cycle phase strongly correlated with the OTU richness (identified
126 absolute number of taxa) and evenness (inverse Simpson index; Fig. 2). Lactating females had
127 a significantly higher OTU richness (<p=0.0001 [Mann-Whitney t-test]) and the bacterial taxa
128 were significantly more evenly distributed (p=0.0001 [Mann-Whitney t-test] than in non-
129 lactating females (Fig. 2A-B). Similarly, animals in a menstruation-like (Phase 3, Table S1)

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130 sexual cycle phase had a significantly higher OTU richness (<p=0.0001 [Kruskal-Wallis test])
131 and were significantly more evenly distributed (p=0.001 [Kruskal-Wallis test]) than animals
132 in the ovulatory (Phase 1) or intermediate phase (Phase 2; Fig. 2C-D). A heatmap of the
133 relative abundance of the 20 most common OTUs shows that lactating animals and animals in
134 menstruation-like sexual phase clustered separately from other animals (Fig. 1). Vaginal
135 bacterial communities from these animals clustered prominently in cluster 1 (C1) and are
136 characterized by different bacterial taxa than the cluster 2 (C2; Fig. 1). Of the ten most
137 abundant OTUs, *Provella*, *Mobiluncus, Porphyromonas* and an unclassified genus of the
138 family *Ruminococcaceae* were significantly different in the lactating and menstruation-like
139 animals (Fig. S2A-B). These cluster differences were confirmed by significant differences in
140 the unweighted UniFrac distances, which are visualized on the principal coordinates plot
141 along axis 1 (23.1%) (Fig. 3A-B). Pairwise AMOVA confirmed that the differential clustering
142 of lactating and menstruating-like animals resulted in significantly different community
143 structures (p=0.001).
144

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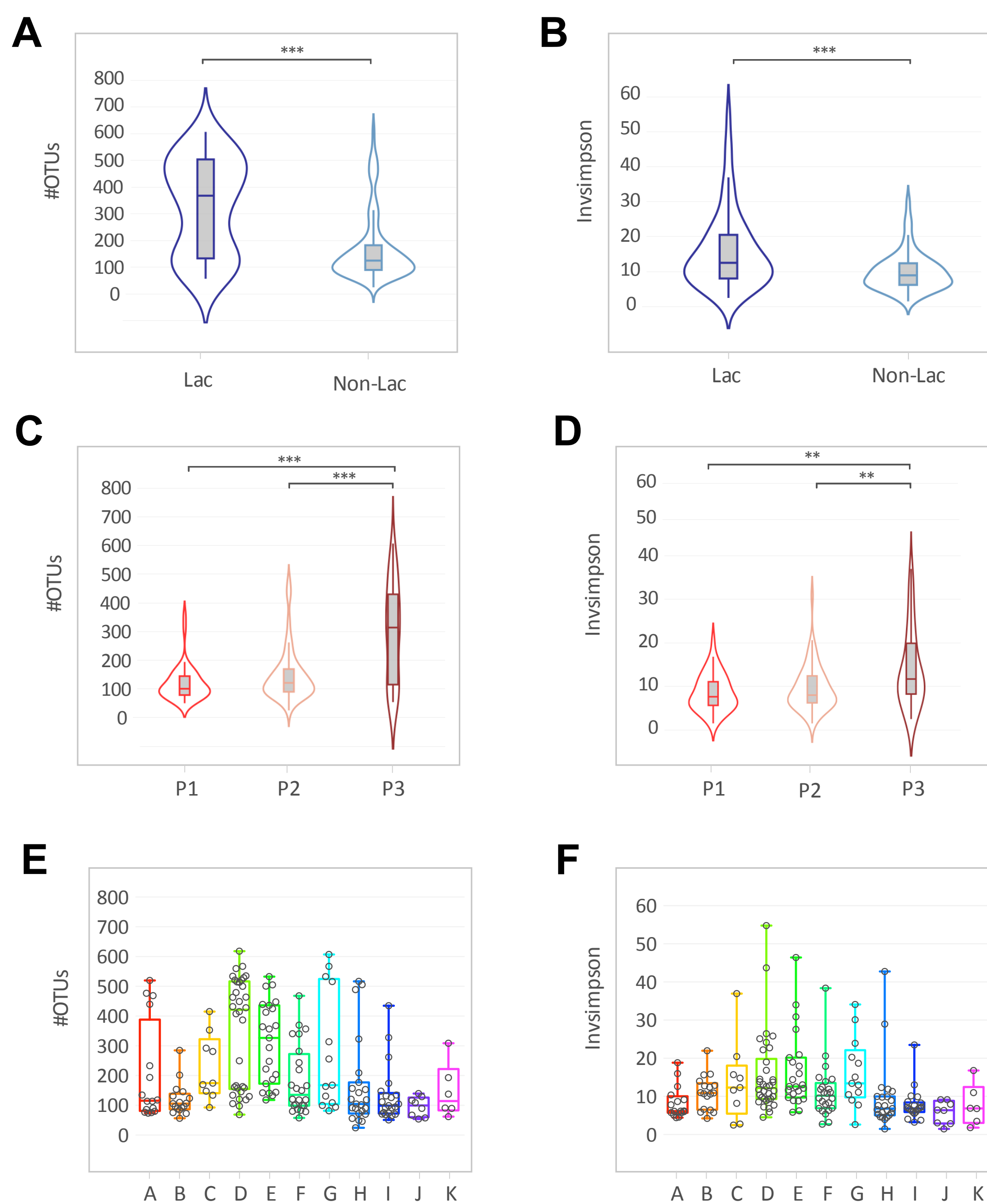

145
146
147 **Fig. 2: Alpha diversity measurements for vaginal microbiome of female rhesus monkeys.**
148 Violin plots of the observed OTUs and InvSimpson index clustered based on (**A/B**) lactation
149 status (Mann-Witney t-test, ***p≤0.0001) and (**C/D**) sexual cycle phases (P1: ovulatory phase,
150 P2: intermediate stage, P3: menstruation-like) (Kruskal-Wallis test, **p≤0.001,
151 ***p≤0.0001). (**E/F**) Boxplots (median ± range) of the observed OTUs and InvSimpson index
152 clustered of breeding groups (groups association: A-K). See Table 1 for sample size
153 composition.

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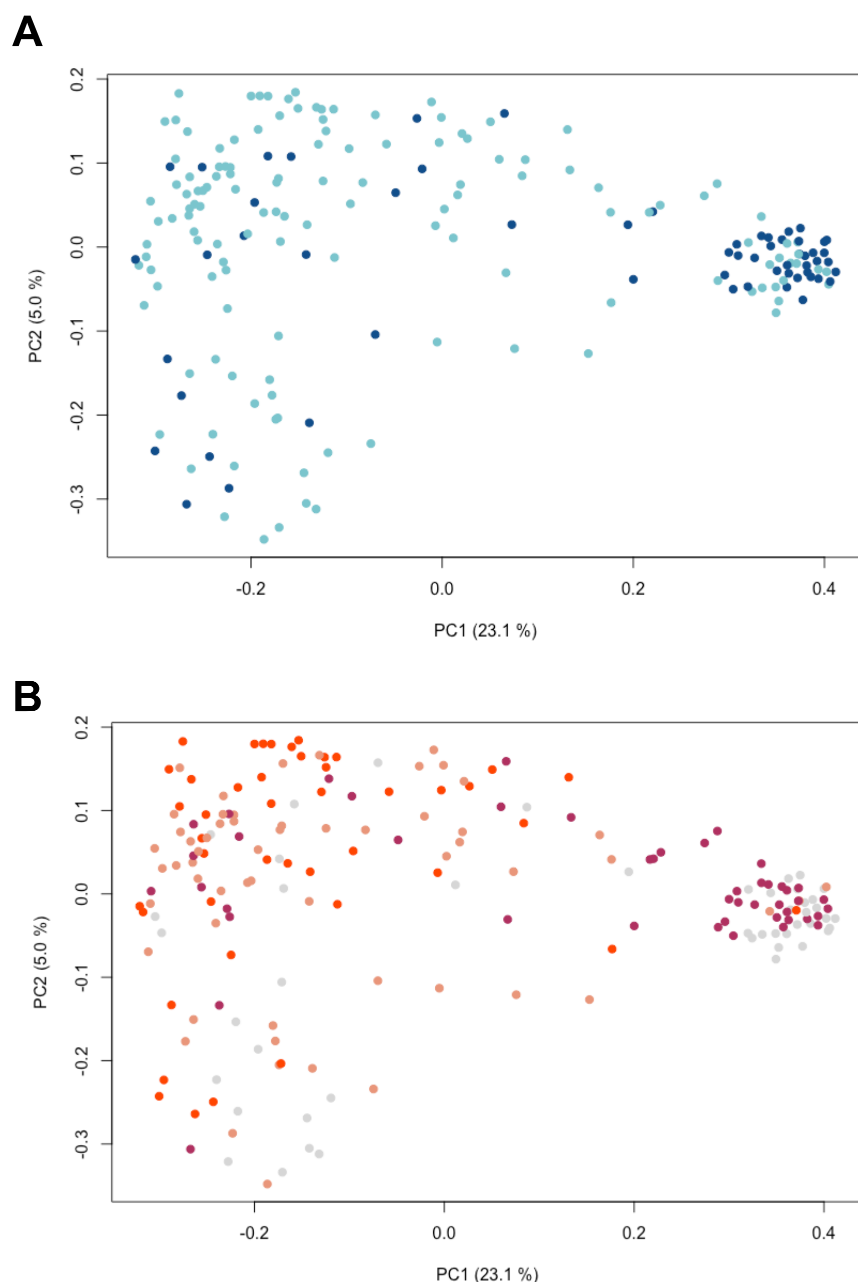

154
155
156 **Fig. 3: The vaginal microbiome of menstruating-like and lactating females clusters**
157 **separately.** Principal coordinates analysis (PCoA) of vaginal samples colored by (**A**)
158 lactation status (lactating (dark blue) and non-lactating (light blue) and (**B**) sexual cycle status
159 (P1: ovulatory phase (red), P2: intermediate stage (pink), P3: menstruation-like (dark red) and
160 NT: not tested (gray)). Distances between samples were calculated using the unweighted
161 UniFrac metrics. See Table 1 for sample size composition. Fig. S5 shows the corresponding
162 PCoA plot classified by group association and age classification.
163
164

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165 In order to examine an additional functional variable of the vaginal microbiota, we tested the
166 vaginal pH at the time of sampling using pH-indicator paper. The mean overall vaginal pH of
167 the sampled animals was found to be 6.4±0.7 (Table 1). The vaginal pH of lactating females
168 was significantly higher than that of non-lactating females (>p=0.0001 [Mann-Whitney t-
169 test], Fig. S3A). Similarly, animals in menstruation-like sexual phase had a higher vaginal pH
170 compared to individuals in the other sexual cycle phase (>p=0.0001 [Kruskal-Wallis test],
171 Fig. S3B).
172
173 ***Breeding groups influence the vaginal microbiome***
174 Aside from lactation status and cycle phase, we examined if the vaginal microbiome is shaped
175 by age and breeding group. Animals were subdivided into juveniles (<5 years), adults (5-19
176 years), and geriatric (>19 years). Age was not found to have an influence on alpha diversity
177 (Fig. S4) and beta diversity (p=0.155 [AMOVA; Fig. S5A). The group association, in
178 contrast, significantly correlate with the OTU richness (>p=0.0001 [Kruskal-Wallis test], Fig.
179 2E) and evenness (>p=0.0001 [Kruskal-Wallis test], Fig. 2F). The breeding group association
180 of each sample can be seen on the heatmap of the 20 most abundant OTUs (Fig. 1).
181 Additionally, we observed a significant difference in unweighted UniFrac distances when
182 considering all breeding groups (<p=0.001 [AMOVA; Fig. S5B). Pairwise comparisons of
183 alpha and beta diversity measurements between individual breeding groups was, however, not
184 significant for all tested groups.
185
186 ***Influence of social rank in adult male rhesus monkeys***
187 We characterized the urethral microbiome of clinically-healthy, reproducing male rhesus
188 monkeys housed in ten different breeding groups (Table 1). Overall, the urethral microbiome
189 of male rhesus monkeys is composed of a diverse community of microbes, with a mean of
190 481.3±127.0 OTUs observed. On a phylum level, *Firmicutes* (54.1±8.3%), *Bacteroidetes*
191 (25.5±9.3%), *Proteobacteria* (9.0±6.7%) and *Actinobacteria* (6.6 ± 3.2%) made up 95% of
192 the identified sequences. The four dominant phyla were present in all 37 samples. On the
193 genus level, the bacterial community is diverse with no single dominating OTU (Fig. 4). The
194 most abundant genus in the male rhesus monkey urethra was *Prevotella* with a mean
195 abundance of 14.4±9.7% followed by *Porphyromona* (7.5±6.6%) and *Ezakiella* (7.3±6.8%).
196
197

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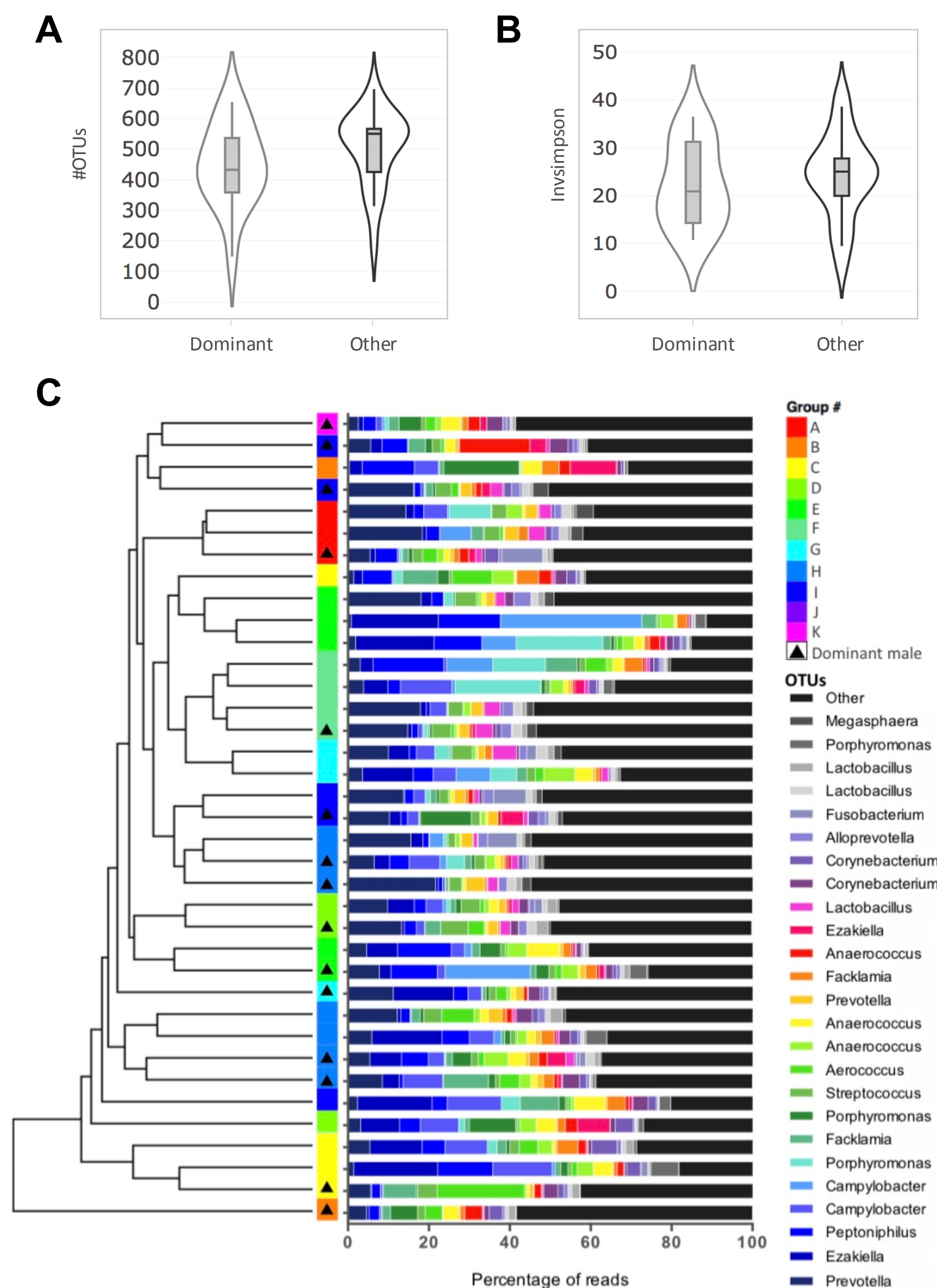

198

199

200 **Fig. 4: The male urethral microbiome of dominant and non-dominant (other) rhesus**
201 **monkeys does not differ significantly.** Violin plot of (**A**) the number of OTUs and (**B**) the
202 inverse Simpson index for dominant and non-dominant (other) males (Mann-Witney t-test).
203 (**C**) UPGMA clustering on unweighted UniFrac including taxa plots showing the relative
204 abundance of the 25 most abundant OTUs in percentage of reads. Genus-level bacterial
205 classification of OTUs shown legend with the percent of sequences that classified with each
206 genus. Group and dominance rank are shown accordingly. See Table 1 for sample size
207 composition.

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208 We examined if a dominance rank in the breeding group shaped the urethral microbiome. The
209 highest-ranking male was classified as the dominant male. Each breeding group had one
210 dominant male with the exception of group H (n=4) and I (n=3), which were further divided
211 into subgroups within a single housing unit. Dominant males neither differed from other
212 males in the OTU richness (p=0.145, [Mann-Whitney test], Fig. 4A) nor the evenness
213 (p=0.453 [Mann-Whitney test], Fig. 4B). Pairwise AMOVA of unweighted UniFrac distances
214 found that dominance rank had no effect on community structure (p=0.123). A dendrogram of
215 unweighted UniFrac distances shows that dominant animals did not cluster separately from
216 other animals (parsimony analysis, p=0.768; Fig. 4C). OTU richness and evenness
217 measurements of each breeding group are shown in Fig. S6. We note here, that the sample
218 size of male animals in each breeding group were low (n=1 to 4 animals). Therefore,
219 statistical analysis was not performed to examine breeding group differences.
220
221 ***Lactating and menstruation female have a more similar microbiome to the male urethra***
222 On the phylum level, *Firmicutes* and *Bacteroidetes* dominated both microbiomes, making up
223 80.1±6.9% in the male urethra and 69.9±17.8% in the vagina. Yet, *Fusobacteria*, the third
224 most abundant phylum in the vagina (14.4±16.1%), only made up 1.9±3.3% in the male
225 urethra. The most abundant genus across the dataset for both, male and female genital
226 microbiome was *Prevotella*. Several OTUs cluster into this bacterial genus and the most
227 abundant *Prevotella* OTUs (mean abundance of 6.0±8.7%) was found in 226 out of 231
228 animals. Other *Prevotella* OTUs were less abundant and only dominant in some samples (Fig.
229 1: vaginal microbiome and Fig. 4: male urethral microbiome).
230 To further examine similarities between the vagina and male urethra, overall OTU
231 richness and evenness was compared. As we previously observed a significant difference in
232 alpha and beta diversity of the vaginal microbiome based on lactation status and sexual cycle
233 phase, these variables were plotted separately (Fig. 5). The male urethra had a significantly
234 higher OTU abundance compared to non-lactating and non-menstruation-like (ovulatory and
235 intermediate phase) animals (<p=0.0001 [Kruskal-Wallis test], Fig. 5A/B). Contrary,
236 menstruation-like (P3) and lactating female rhesus monkeys showed no significant difference
237 in the number of OTUs compared to the male urethra microbiome (>p=0.05 [Kruskal-Wallis
238 test], Fig. 5A/B). Similarly, inverse Simpson index measurements were significantly different
239 between males and non-lactating and non-menstruation-like (ovulatory (P1) and intermediate
240 phase (P2)) animals (<p=0.0001 [Kruskal-Wallis test], Fig. 5C/D). Inverse Simpson index
241 measurements were not significantly different between males and lactating and menstruation-

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242 like animals (>p=0.05 [Kruskal-Wallis test], Fig. 5C/D). To examine, if the trend in the alpha
243 diversity could be observed in the overall bacterial composition, pairwise unweighted
244 UniFrac distances were calculated between the male urethral microbiome and the vaginal
245 microbiome. More similar microbiomes resulted in a smaller calculated UniFrac distances and
246 vise versa. The UniFrac distances were grouped in violin plots based on either lactation status
247 (Fig. 5E) or sexual cycle phase (Fig. 5F). We found that the bacterial composition of the
248 vaginal microbiome of lactating and menstruating-like animals (P3) was significantly more
249 similar to that of the male urethra microbiome (Fig. 5E/F).
250

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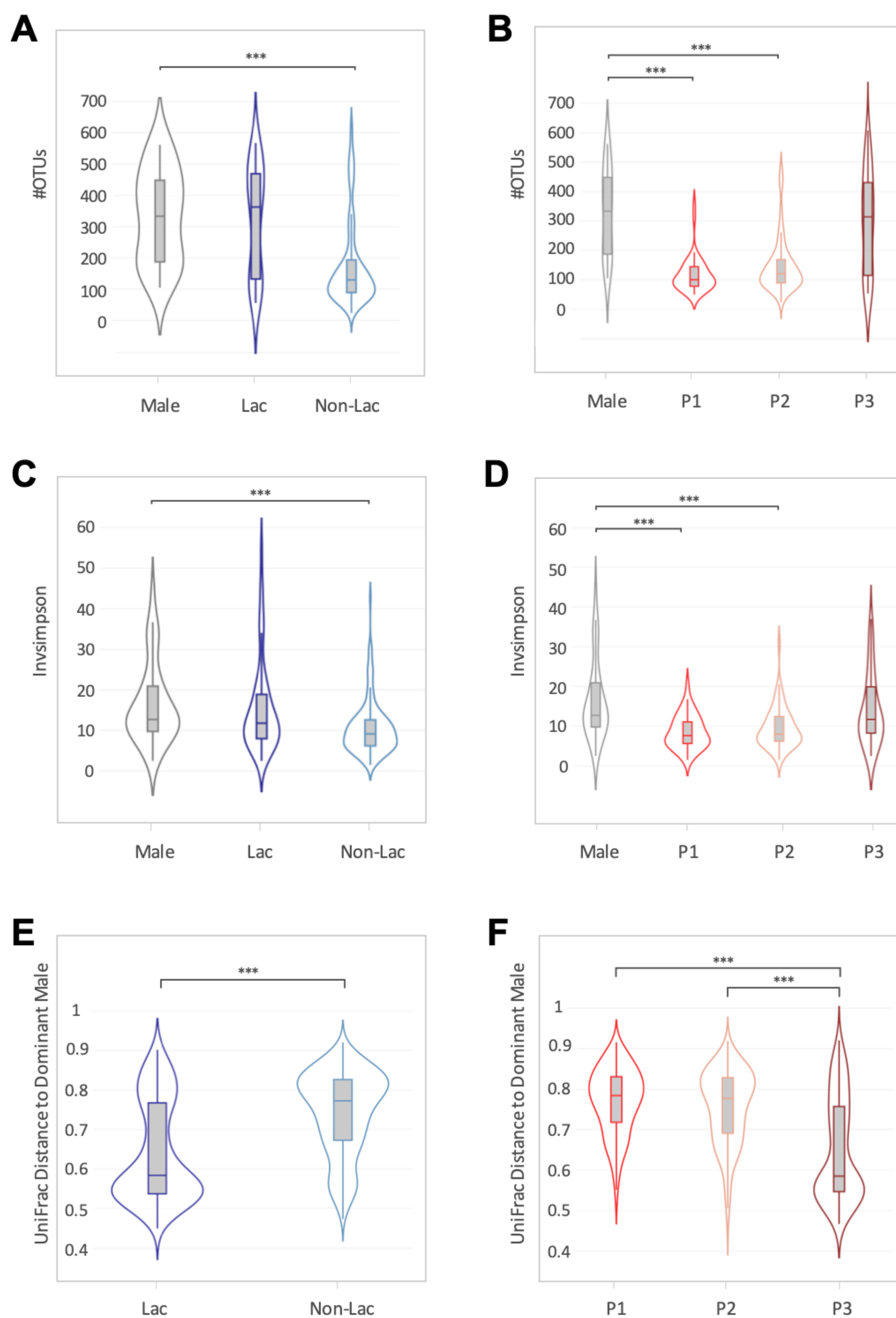

251
252

253 **Fig. 5: The vaginal microbiome of lactating and menstruating females is more similar to**
254 **the urethral microbiome.** Violin plot of the number of OTUs (**A**) in the male urethra and
255 vaginal microbiome of lactating and non-lactating females and (**B**) in the male urethra and
256 vaginal microbiome of females in three sexual cycle phases (P1: ovulatory phase, P2:
257 intermediate stage, P3: menstruation-like) (Kruskal-Wallis test, ***p≤0.0001). Violin plot of

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258 the inverse Simpson index for (**C**) male urethra and vaginal microbiome of lactating and non-
259 lactating females and (**D**) male urethra and vaginal microbiome of females in three sexual
260 cycle phases (Kruskal-Wallis test, ***p≤0.0001). (**E/F**) Violin representations showing
261 unweight UniFrac Distance of each female to the dominant male in each group. Data is
262 plotted by (**E**) lactation status (Kruskal-Wallis test, ***p≤0.0001) and (**F**) sexual cycle phase
263 (Mann-Witney t-test, ***p≤0.0001). See Table 1 for sample size composition.
264

265 ***Cage-mates are more similar in their genital microbiome***

266 In order to assess if sexual contact shapes the genital microbiome of the captive rhesus
267 monkeys, pairwise unweighted UniFrac distances were calculated between the male urethral
268 microbiome of the dominant male in each group and the vaginal microbiome. Females and
269 males of the same breeding group were considered cage-mates and thus potential sexual
270 partners. UniFrac distances were grouped into violin plots as 'cage-mates' or from 'other
271 breeding groups' (no sexual contact possible) (Fig. S7A). Cage-mates were found to be
272 significantly more similar in the bacterial composition compared to non-cage-mates
273 (<p=0.0001 [Mann-Whitney test], Fig. 7A). As we observed a significant difference in
274 lactation status and sexual cycle phase, these variables were additionally plotted in separate
275 paired-violin plots to examine cage-mate differences for each group (Fig. S7B/C). Cage-
276 mates were found to be significantly more similar in the bacterial composition for lactation,
277 menstruation-like and ovulatory phase animals (Fig. S7B/C). Cage-mate similarity was not
278 observed for the non-lactating group or for animals in an intermediate sexual phase (P2; Fig.
279 S7B/C).
280

281 **Discussion**

282 Considering the effect that microbiome variation can have on disease acquisition and outcome
283 (*1*), we examined endogenous and exogenous factors that influence the urogenital microbiome
284 of captive rhesus monkeys. The inclusion of appropriate controls (Fig. S1) and the large
285 sample size confer confidence in our study. However, based on our cross-sectional study
286 design we were limited in drawing causal relationships between factors and variations in the
287 genital microbiome. Nevertheless, our results urge for the inclusion of microbiome analysis in
288 the selection and experimental use of rhesus monkeys as indicated by the differences between
289 the vaginal microbiome during lactation and sexual cycles phases.

290 We showed that during the endocrine state, which causes the female to lactate and
291 menstruate the bacterial composition shifts towards a more diverse community (Fig. 1-3). As

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292 reported previously, we confirmed that the mean vaginal pH of rhesus monkeys (6.4±0.7) is
293 significantly higher than that found in humans (*2*) (Fig. S3). Instead of the *Lactobacillus*-
294 dominance observed in women (reviewed by (*18*)), the vaginal microbiome of captive and
295 wild NHPs harbor a more diverse set of bacteria (Fig. 1) (*2, 11, 12, 15*). Our study shows that
296 in captive rhesus monkeys the already diverse bacterial community shifts to an even more
297 diverse and significantly different bacterial composition during lactation and the
298 menstruation-like phase (Fig. 2-3). There has been some discussion if the sexual cycle
299 influences the vaginal microbiome of captive NHPs. While a previous study on captive
300 baboons (*Papio anubis*) found no difference in the vaginal microbiome of cycling females
301 (*15*), a recent study in wild baboons (*Papio cynocephalus*) reported that the ovarian cycle
302 phase and the reproductive state shaped the vaginal microbiome (*12*). Both of these studies
303 used visual assessment of perivulvar swellings to determine the sexual cycle phase (*12, 15*).
304 Inconsistent classification of these phases in the two studies in combination with a low
305 sample sizes may explain the difference in the outcome of both studies. Instead of using
306 perivulvar swellings, we performed vaginal exfoliative cytology to classify the animals into
307 three sexual cycle phases ((*19, 20*); Table S1)). Vaginal exfoliative cytology reflects the
308 current state of the vaginal epithelium and therefore serves as a reliable marker for the sexual
309 cycle phase (*19, 20*). The even distribution of all three sexual cycle phases in non-lactating
310 rhesus monkeys (Table S1) is indicative of a healthy reproductive community. Using cytology
311 as a marker of sexual cycle phase, this study supports *Miller et al*.'s finding that ovarian cycle
312 phase (menstruation-like) and reproductive state (lactation) shifts the vaginal microbiome in
313 NHPs (*12*). Similar changes have been reported in temporal and cross-section studies in
314 women during menstruation and post-partum (*13, 21*), where it has been demonstrated that
315 the vaginal microbiome shifted from a *Lactobacillus*-dominant state towards a more diverse
316 bacterial composition (*13, 21*). Despite the remarkable differences in bacterial species
317 composition of the rhesus monkeys and human vaginal microbiome, it is interesting that
318 similar factors (e.g. hormonal changes) seem to influence the vaginal microbiome. This is
319 supported by our finding that the observed changes in bacterial vaginal diversity in the rhesus
320 monkeys coincide with changes in the pH, a functional measurement of the vaginal
321 ecosystem. Understanding the causal relationships that regulate and control changes in
322 vaginal ecosystem is a current challenge for both NHP and human microbiome studies
323 (reviewed by (*22*)) and warrants further investigation.

324 It has been proposed that hormonal fluctuations during the sexual cycle, pregnancy
325 and post-partum shape the vaginal microbiome (reviewed by (*18*)). Both lactation and

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326 menstruation are marked by hormonal changes in the vagina, which may be indirect or direct
327 driving factors for the shift in vaginal microbiome observed in this study (Fig. 1-3). Studies
328 on SIV susceptibility in rhesus monkeys have shown that hormone treatment can lead to an
329 altered susceptibility (*3, 9*). During high levels of estrogen, changes in the vaginal epithelium,
330 including changes in vaginal microenvironment, may have a protective effect (*3*). The less
331 diverse vaginal microbiome and lower overall pH (Fig. S3) found in this study during
332 ovulatory phase supports an important role of the vaginal microenvironment in infectious
333 disease acquisition. Further investigations are necessary to examine the causal relationship
334 between hormone levels, changes in the NHP vaginal microbiome, and susceptibility to
335 pathogens. However, it has become clear, that a more holistic understanding on host-
336 pathogens interactions is required for the interpretation of animal experiments as host factors
337 can influence the microbiome and vice versa (reviewed by (*1*)).

338 We examined the male urethral microbiome of the rhesus monkeys to further compare
339 the genital microbiome of females and males in a single breeding unit. To our knowledge,
340 there has been no studies on the urethral microbiome of wild or captive NHPs to date. Four
341 bacterial phyla, *Firmicutes, Bacteroidetes*, *Proteobacteria,* and *Actinobacteria*, compose the
342 majority of identified sequences in the urethra. On the phylum level, the urethral microbiome
343 of the male rhesus monkeys were similar to that reported in humans with *Firmicutes* making
344 up the largest proportion (*23*). In our male animals, notable urethral taxa include *Prevotella*,
345 *Porphyromona*, and *Ezakiella*, have all been previously associated with the urinary tract
346 microbiome of adult men (*23-25*). *Prevotella* has been previously detected in the genital
347 microbiome of healthy female rhesus monkeys indicating that this genus plays a residential
348 role in the rhesus monkeys genital microbiome (*10*). In humans, some species of *Prevotella*
349 have been associated with disease states (e.g., bacterial vaginosis (*26*)) while other species
350 can be found in clinically healthy women (e.g., post-partum, (*21*)). Identifying the role of
351 *Prevotella* in NHPs may assist in a better functional understanding of the genital ecosystem.
352 For the urethral microbiome, it is difficult to compare the prevalence of *Prevotella* in the male
353 rhesus monkey to other studies, as there is currently no consensus on the core urethral
354 microbiome, even in humans (*25*). As a result, large scale investigations need to be performed
355 to study the male urogenital microbiome including factors that influence this unique
356 ecosystem in health and disease (*25*).

357 It has been hypothesized that sexual exposures can alter the composition of the genital
358 flora (*13, 16, 25*). A recent study on sexual partners with bacterial vaginosis (BV), showed
359 that women with BV were significantly more similar to the urogenital microbiome of their

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360 partner (*17*). To test if sexual contact affected the genital microbiome of NHPs, we first
361 examined if dominance rank in captive rhesus monkeys shaped the male urethral microbiome.
362 Breeding groups in this study contained a single breeding male, who monopolized the cage-
363 mates in estrus. We did not find that dominance rank shaped the male urethral microbiome
364 (Fig. 4). This may be due to the fact that non-dominant juvenile rhesus monkeys already
365 engage in socio-sexual mounting as a form of play (*27*). Contrary to our findings, sexual
366 history in healthy adolescent men has been reported to be a possible determinant of the
367 urogenital microbiota (*16*). Known sexually transmitted bacteria and taxa associated with the
368 urethral tract of adult men (*23*), were observed rarely in adolescent men (*16*). To further study
369 the effect of sexual contact, we examined the similarity of the genital microbiome in cage-
370 mates (alpha male to females in the same breeding group). We found that overall, cage-mates
371 were significantly more similar to each other compared to non-cage-mates (Fig. S7A). When
372 subdividing cage-mate by lactation status and sexual cycle, the observed cage-mate effect was
373 not seen for non-lactation and intermediate sexual phase animals (Fig. S7B-C). This may be
374 due to an inappropriate subsampling of these two groups. For example, the intermediate
375 sexual phase classification used in the EVC may represent both, proliferative phase and
376 secretory phase, and is therefore an oversimplification. This highlights the limitation of this
377 cross-sectional study in assessing cage-mate similarities. A controlled temporal study is
378 necessary to examine the effect of sexual contract in NHP breeding groups. NHPs can be an
379 advantages model to further examine microbiome similarities in sexual partners (in health and
380 disease) as sexual contact is easily observed and controlled.
381 A surprising finding of our study was that independent of breeding group association,
382 the bacterial composition of lactating monkeys and/or those in the menstruation-like sexual
383 phase were more similar to the microbiome of the male urethra (Fig. 5). As females in a
384 menstruation-like sexual phase are less attractive to male rhesus monkeys, we presume that
385 the similarity is not caused by recent sexual contact. A possible explanation for this finding is
386 that the altered hormonal state allows otherwise more-suppressed bacteria to dominate the
387 microflora. To understand the cause of the vaginal microbiome shift towards the male urethra
388 microbiome, controlled temporal experiments in NHP would be necessary. Interestingly, a
389 temporal study in humans has shown that the vaginal microbiome post-partum shifts towards
390 the gut microbiome (*21*). The study was able to show that the shift towards the gut
391 microbiome persisted for multiple months and was independent of delivery method (vaginal
392 vs. caesarean). These findings support the notion that during changes in the genital ecosystem
393 (e.g., shifts in hormones), the vagina is more susceptible to ‘foreign’ bacteria. This potentially

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394 altered susceptibility should be carefully considered when performing vaginal inoculations in
395 NHPs for future experiments (e.g., HIV).
396 We found that breeding groups can have an effect on the vaginal microbiome (Fig. 2).
397 Breeding group similarities could be influenced by various factors including host genetics
398 (*28*), differences in group size or cage effects (*29*). Many of these factors could not be
399 properly examined in this study and require planned and controlled animal experiments. In
400 mice, it has been shown that animals kept in the same cage become more similar in
401 microbiome composition over time (*29*). This effect could be studied in captive NHPs by
402 examining microbiome changes in various ecological niches (genital, skin, fecal) during cage
403 transfers. A cage effect in NHPs could have major implications for the use of NHPs as
404 translational animal models. A better understanding of the NHP microbiome could therefore
405 refine animal selection for animal experiments where a higher standardization can lead to
406 reduced animal numbers (*3, 9*). The inclusion of appropriate controls in microbiome studies
407 cannot be stressed enough (*30*). Especially low abundance microbiomes like the urethral
408 microbiome are vulnerable to contaminations during sampling and laboratory analysis (*31*).
409 The inclusion of blank control samples, especially at the site of sample collection, is essential
410 and should be understood as Good Laboratory and Scientific Practice (Fig. S1). Only well-
411 planned and controlled microbiome studies on NHPs are capable of effectively reduce and
412 refine NHP numbers in translational animal models and providing a better understanding of
413 factors that influence microbiomes of NHPs.
414
415 **Methods**
416 ***Ethical statement.*** All samples included into this study were obtained from clinically healthy
417 rhesus monkeys that underwent the mandatory annual health check at the German Primate
418 Center between June 2016 and May 2017. Animals were not purposely immobilized to collect
419 samples for this study. Swabs were taken as part of a routine annual health monitoring and
420 tuberculosis screening. Animal were short-term immobilized by trained veterinarians who
421 checked and documented the general health condition of each individual. Sampling included
422 the collection of blood, oral and genital swab samples. The use of the samples was reviewed
423 and approved by the animal welfare and ethics committee of the German Primate Center (EC
424 No. 1-16). All work steps involving the handling of live animals followed the rules of 'Good
425 Veterinary Practice'.
426

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427 ***Study Design and Animals.*** Urethral swabs of 37 male and vaginal swabs of 194 female
428 rhesus monkeys were collected. A cross-sectional study design was applied. Samples from
429 apparently pregnant individuals, clinically diseased animals, or animals that received medical
430 treatment within the last 6 months were excluded from analysis. Moreover, we excluded
431 samples from animals below the age of three. Data file S1 provides a detailed overview on the
432 samples analyzed in this study as well as the respective NCBI Sequence Read Archive
433 numbers. Lot numbers for consumables were kept consistent and are reported in the
434 Supplementary Material (Table S2).
435
436 ***Swab Sample Collection***. Immobilized female rhesus monkeys were placed in dorsal
437 recumbency and the area around the vulva was cleaned using 70% ethanol. To facilitate
438 sampling, a sterile silicon tube, 15 mm diameter and 40 mm length, was used to avoid swab
439 contamination with skin or fecal material. A flocked swab (FLOQSwabs, Copan Improve
440 Diagnostics) was moistened using a single drop of sterile physiological saline solution (WDT
441 eG) and was subsequently inserted midway into the vaginal canal. Subsequently the swab was
442 rotated 20-times on the dorsal wall before it was gently removed and transferred into 500 μl
443 of custom-made lysis buffer (10mM Tris, pH 8.0, 0.1M EDTA, pH 8.0 and 0.5% SDS).
444 Samples were kept on ice until transported to the inhouse laboratory facilities where they
445 were stored at -80°C (*30*).
446 An additional swab was collected to perform an EVC. Briefly, the swab was rolled
447 onto a microscope glass slide after which it was allowed to air-dry. Slides were then stained
448 with a Romanowski stain (Diff-Quik) and subsequently examined under the microscope by
449 two independent investigators (*19*). Cytological scoring was performed as previously
450 described by *McLennan et al*. (*20*). The maturation index was calculated by counting 100
451 representative epithelial cells, which were scored according to their cell type. Briefly,
452 parabasal cells were assigned a value of 0, intermediate cells a value of 0.5, and superficial
453 cells a value of 1. Based on the cumulative maturation score, the animals were categorized
454 into three stages (ovulatory phase (P1), intermediate phase (P2), and menstruation-like phase
455 (P3); see Supplementary Table 2).
456 The vaginal pH was measured using a swab which was inserted midway into the
457 vagina and then rolled onto a pH-indicator paper (Merck & Co, Inc.). The vaginal pH was
458 scored by two independent researchers following the manufacturer's instructions using a scale
459 ranging from 5.5 to 9.0.
460

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461 Immobilized male rhesus monkeys were placed in ventral recumbency and sampled for
462 urethral swabs. A minitip FLOQ swabs (Copan Improve Diagnostics) was moistened using
463 sterile physiological saline solution and subsequently inserted 1-2 cm into the urethra of the
464 animal. Subsequent handling of the samples was identical to the procedure described for
465 vaginal swab samples.
466 Suitable precautions were taken during sample collection to avoid microbial
467 contamination. As a sample collection control, a FLOQ swab with a single drop of sterile
468 physiological saline solution was immediately transferred into a 500 μl custom-made lysis
469 buffer at the breeding facility at the time of sampling.
470
471 ***DNA Extraction***. We used the QIAamp Mini Kit (Qiagen GmbH) to extract bacterial DNA.
472 This kit was previously validated for microbial analysis of swab material (*30*). Briefly,
473 proteinase K (50mg/µl) was added and the samples were incubated overnight at 56°C at
474 600 rpm (Thermomix comfort, Eppendorf). Appropriate amounts of AL buffer (Qiagen
475 GmbH) and ethanol were added. The DNA was subsequently purified from the lysate using
476 the spin columns provided in the kit. Extracted DNA was eluted in 75µl Microbial DNA-Free
477 water (Qiagen GmbH). Suitable precautions were taken during sample handling and
478 processing in the laboratory to limit microbial contamination and maintain consistency during
479 all procedures. The order of sample processing was randomized to avoid handling bias. As a
480 laboratory analysis collection control, a FLOQ swab was transferred into a 500 μl custom-
481 made lysis buffer under the DNA extraction bench at the time the rhesus monkey samples
482 were handled.
483
484 ***16S ribosomal RNA gene sequencing.*** The universal primers 515F and 806R, which were
485 adapted with linker regions and barcode sequences, were used to amplify the V4 region of the
486 16S ribosomal RNA (16S rRNA) gene (*32*). Phusion Hot Start II High-Fidelity DNA
487 Polymerase (Thermo Fisher Scientific), which has been previously validated for the use in
488 microbiome studies (*30*), was used to amplify each sample in triplets. PCR reactions consisted
489 of 12.5µl of 2x PCR master mix, 8µl of Microbial DNA-Free water (Qiagen GmbH), 1.25µl
490 of each primer (0.5mM each, Metabion) and 2µl of template in a total reaction volume of
491 25µl. PCR cycling conditions comprised of a pre-denaturation step of 30s at 98°C, followed
492 by 30 cycles of 98°C for 10s, 55°C for 15s and 72°C for 60s, as well as a final 10 min
493 extension step at 72°C. A blank control (Microbial DNA-Free water) and a mock control
494 sample (Microbial mock community, HM-280, Biodefense and Emerging Infectious Research

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495 (BEI) Resources, Manassas, USA) were included in 16S rRNA gene amplification. The
496 amplicon triplets were pooled, purified using 0.7x AMPure XP beads (Beckman Coulter), and
497 quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Subsequently, we
498 verified the amplicon integrity for a representative number of eleven samples using the
499 BioAnalyzer 2000 (Agilent). Equimolar amounts (10nM) of sample amplicon and maximum
500 volume of control samples (5µl) were pooled prior to sequencing. Illumina MiSeq 2x250bp
501 paired-end sequencing (Illumina V2 chemistry) was performed in the Transcriptome and
502 Genome Analysis Laboratory at the University of Göttingen in accordance with published
503 guidelines (*32*). All generated read files are available at the NCBI Sequence Read Archive
504 (PRJNA521516).
505
506 ***Data processing and analysis.*** The sequencing reads were processed using the mothur
507 software package (v.1.39.5) (*33*). According to the MiSeq SOP (*33*), contigs were assembled,
508 sequences were quality filtered, and PCR artifacts were removed. The SILVA bacterial
509 reference database (*34*) was used to align the sequences and OTUs were assigned based on
510 97% sequence similarity. Cross-sample singletons and poorly aligned sequences were
511 removed. The seq.error command was used to determine the error rate and the mock
512 community was eliminated from the dataset. Due to low read numbers, control sample reads
513 were excluded from the dataset and analyzed separately.
514 To examine differences in the microbial community structure, alpha (species richness
515 within a single sample) and beta diversity (microbial community diversity between samples)
516 was calculated. As alpha diversity measurements, we determined the number of observed
517 OTUs and calculated the inverse Simpson Metrix using the summary.single command in
518 mothur. Beta diversity was determined using unweighted UniFrac metrics (*35*). The
519 dissimilarity matrix was visualized using Principal Coordinates Analysis (PCoA) and a
520 Newick formatted dendrogram (visualized in FigTree v.1.4.2,
521 http://tree.bio.ed.ac.uk/software/figtree/). ClustVis tool (https://biit.cs.ut.ee/clustvis/) was
522 used to create a heatmap of the relative abundance of bacterial taxa (*36*). Violin plots (R
523 package plot.ly) and box plots (GraphPad Prism 6) were used to visualize data points for
524 different variables.
525
526 ***Statistical analysis.*** The statistical significance of the pooled data was analyzed in GraphPad
527 Prism 6 (GraphPad software) and the R package 'vegan'. Whenever appropriate, we tested for
528 normality distribution of the data using the Kolmogorov-Smirnov normality test. The

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529 significance in alpha diversity and pair-wise beta diversity between two or more groups was
530 tested using the non-parametric Mann-Whitney-U or Kruskal-Wallis tests including
531 correction for multiple testing using Dunn's post hoc tests. Differences in community
532 structure based on age of animals, group association, lactation status and dominance rank was
533 tested using analysis of molecular variance (AMOVA, 1,000 permutations) in mothur (*37*).
534 Principal Coordinates Analysis (PCoA) plots of unweighted UniFrac metrics and UPGMA-
535 clustered dendrograms (unweighted UniFrac metrics) were used to visualize data points.
536 Differences in the ten most abundant OTUs in vaginal samples were assessed using the
537 metastats command in mothur (*38*). p-values for differences in individual OTUs were
538 corrected for multiple comparisons using Bonferroni correction. Values of $p < 0.05$ were
539 considered statistically significant.
540

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