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Low *Lactobacilli* Abundance and Polymicrobial Diversity in the Lower Reproductive Tract of Female Rhesus Monkeys Do Not Compromise their Reproductive Success

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Abstract

The lower reproductive tract of nonhuman primates is colonized with a diverse microbiota, resembling bacterial vaginosis (BV), a gynecological condition associated with negative reproductive outcomes in women. Our 4 aims were to: 1) assess the prevalence of low *Lactobacilli* and a BV-like profile in female rhesus monkeys, 2) quantify cytokines in their cervicovaginal fluid (CVF), 3) examine the composition and structure of their mucosal microbiota with culture-independent sequencing methods, and 4) evaluate the potential influence on reproductive success. CVF specimens were obtained from 27 female rhesus monkeys for Gram's staining, and to determine acidity (pH), and quantify proinflammatory cytokines. Based on Nugent's classification, 40% had a score of 7 or higher, which would be indicative of BV in women. Nugent scores were significantly correlated with the pH of the CVF. Interleukin-1 β was present at high concentrations, but not further elevated by high Nugent scores. Vaginal swabs were obtained from 8 additional females to determine microbial diversity by rRNA gene amplicon sequencing. At the phylum level, the *Firmicutes/Bacteroidetes* ratio was low. The relative abundance of *Lactobacilli* was also low (between 3–17%), and 11 other genera were present at >1%. However, neither the microbial diversity in the community structure, nor high Nugent scores, was associated with reduced fecundity. Female monkeys provide an opportunity to understand how reproductive success can be sustained in the presence of a diverse polymicrobial community in the reproductive tract.

Keywords

rhesus macaque; *Lactobacilli*; interleukin-1; bacterial vaginosis; reproductive success

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INTRODUCTION

All surfaces of the body are colonized with bacteria, and it has become increasingly clear that this host/microbial relationship is critical for health, while dysbiosis, or an abnormal community composition, is often associated with disease [Council et al., 2015]. Most research has focused on the gut microbiota because of its particularly high abundance and significance for host digestion and metabolism. However, the resident bacteria of the reproductive tract also play an important role, influencing the acidity of the cervicovaginal fluid (CVF), inflammatory and immune reactions, and reproductive success in women [Eloe-Fadrosh and Rasko, 2013]. For those who study nonhuman primates, it is of further interest that there is a very dramatic difference between the vaginal bacteria found in female monkeys and apes as compared to the microbial profiles of healthy women [Stumpf et al., 2013]. In contrast to the typical predominance of just one genus, *Lactobacilli*, in women [Ravel et al., 2011], a more diverse polymicrobial community structure is commonly found in the lower reproductive tract of all nonhuman primates that have been studied [Yildirim et al., 2014].

Lactobacilli do not predominate in the lower reproductive tract of chimpanzees, our closest relative or in Old World monkeys including the rhesus macaque assessed in our studies [Spear et al., 2010; Stumpf et al., 2013]. Both culture-based and DNA sequencing methods indicate a more diverse bacterial community is typical, including in baboons [Uchihashi et al., 2015]. In keeping with these findings, the normal pH of the CVF in macaques is 6.0–7.0 [Lagenaur et al., 2011], more basic than the acidic range of 3.0–4.4 for premenopausal women without bacterial vaginosis (BV) [Mirmonsef et al. 2012]. In addition, the concentration of lactic acid, which is produced by *Lactobacilli*, is low in monkey CVF, more comparable to the lower levels in women with BV [Nelson et al., 2015]. Thus, female monkeys appear to be a natural model for evaluating the correlates and consequences of a diverse microbial profile in the lower reproductive tract. For example, *Lactobacilli* serve a protective barrier function, competing with other taxa, and when *Lactobacilli* are low, it enables pathogenic bacteria to opportunistically invade and overgrow [Borges et al., 2014; Verstraeten et al., 2009]. The presence of taxa associated with gynecological dysfunction in women, including *Mobiluncus*, *Sneathia*, *Porphyromonas*, *Prevotella* and *Gardnerella*, are reported to be common in specimens from female monkeys [Rivera et al., 2010; Spear et al., 2012]. Our DNA sequencing-based approach was designed to identify and measure the abundance of these taxa.

Soluble inflammatory and immune factors secreted by the host help to promote a healthy microbial balance. Specifically, cytokines in the CVF regulate the beneficial bacteria as well as orchestrate the defense against invasive bacterial and viral pathogens [Doerflinger et al., 2014; Mitchell & Marrazzo, 2014]. Proinflammatory cytokines, including interleukin-1 β , have been reported to be elevated in women with BV, and were assessed in our female monkeys [Beiji et al., 2007; Kyongo et al., 2012]. The aim was to replicate reports of high cytokine levels in monkey CVF, and to address the hypothesis that there might be an association with indicators of dysbiosis, including high Nugent scores [Spear et al., 2012]. The Nugent score is a traditional test used in gynecological practice to diagnose BV, and is based on a Gram stain scoring system [Nugent et al., 1991]. Prior assessments of female

monkeys indicated that their specimens often receive high scores [Doyle et al., 1991; Hu et al., 2015]. The aim was to determine the prevalence of this condition in an established breeding colony of rhesus monkeys, and to address the hypothesis that it might be associated with elevated cytokine levels indicative of inflammation.

Recent articles on the vaginal microbiota and CVF of nonhuman primates often focus on the significance for sexually transmitted diseases, especially the individual variation that can contribute to a greater susceptibility to infection with simian immunodeficiency virus [Spear et al., 2010; Mirmonsef et al., 2012b]. However, one paper took a broader ecological and evolutionary approach and compared 8 different primate species [Yildirim et al., 2014]. The authors concluded that species differences in microbial profiles reflected reproductive strategies, including whether a female was promiscuous and mated with multiple partners. Because that analysis was conducted primarily with primates living in natural settings, it was not possible to control for hygiene or the frequency of mating activity. Thus, it was of interest to evaluate female monkeys living in a laboratory setting with standardized husbandry and breeding protocols. We were able to exclude one potential confound, recent sexual behavior, which can affect both the vaginal microbiota and acidity of the CVF. Complete records of mating and births were reviewed to determine if a high Nugent score or a relatively low abundance of *Lactobacilli* was associated with reduced fecundity and poor pregnancy outcomes. Specifically, the question was whether a female monkey's microbial profile would provide insight into the rapidity with which she typically conceived, the occurrence of problematic pregnancy outcomes, and the number of viable offspring birthed by the time she reached mid-adulthood.

METHODS

Animals

Non-pregnant healthy rhesus monkeys (*Macaca mulatta*) were randomly selected from a population of 150+ females in a large breeding colony. This colony was established in the 1950s with monkeys derived originally from India and has been closed since the 1980s. Twenty-seven multiparous adults, 11.5 ± 0.9 years (range: 3–19 years), were assigned to the first study to assess vaginosis, cytokines and reproductive success. Eight additional females, 11.0 ± 1.8 years (range: 7–21 years), were assessed in a second study to determine the ratio of *Firmicutes* to *Bacteroidetes* at the phylum level, the relative abundance of *Lactobacilli* and their relationship to other genera, and reproductive success. All monkeys were born and raised in a controlled indoor facility and housed in stainless steel cages, which were cleaned daily and completely disinfected every 2 weeks. To exclude an influence of recent sexual behavior and contact with males, all females were assessed in their current stable housing conditions. In both studies, approximately half were housed individually proximal to other females; the remainder lived as a dyadic pair with another female. They were fed a standardized diet comprised of biscuits manufactured by a commercial vendor (PMI international 5L1Q, St. Louis, MO), and supplemented with fruits and vegetables.

Ethics Statement

Experimental and husbandry procedures were approved by the Animal Care and Use Committee (ACUC) at the University of Wisconsin-Madison. Specimen processing was approved by the Office of Biological Safety. This research adhered to the principles of the American Society of Primatologists regarding the ethical treatment of primates.

Sample Collection and Processing

Samples were collected during the follicular phase in Study 1 to control for a possible influence of the menstrual cycle [Yu et al., 2009]. Monkeys were sedated briefly with ketamine (10 mg/kg) to minimize the disturbance associated with the specimen collection procedures. Cervicovaginal fluid (CVF) was obtained with sterile swabs, which were used for the microbiological analysis, pH determination, and the cytokine assay. For the microbial assessment, two culture swabs (BBL CultureSwab Plus) were inserted into the vagina for 5 sec and then sent immediately to a diagnostic lab at the School of Veterinary Medicine at UW-Madison. A second swab (BBL CultureSwab) was used for determining CVF acidity by depositing the specimen on a pH strip and reading within 1 min. Finally, for quantifying cytokine levels, two swabs (WECK-CEL) moistened with 50 μ L of phosphate buffered saline (PBS) were inserted for 5 min to absorb CVF. The swabs were weighed, CVF obtained via centrifugation, and aliquots stored at -60°C until the cytokine assay.

For Study 2, additional vaginal specimens were obtained with culture swabs (BBL CultureSwab) from 8 conscious animals and frozen at -60°C . Genomic DNA was extracted and 16S rRNA gene amplicons (V4 region) sequenced on the Illumina MiSeq platform.

Assessment of Vaginitis

Swabs were processed by Gram staining to generate a Nugent score. Briefly, the swabs were smeared directly on glass slides and air-dried for a standard Gram stain. A technician blinded to other data about the animals evaluated the results according to traditional criteria [Nugent et al., 1991]. Monkeys were assigned a score between 0–10 based on the presence of *Lactobacillus* morphotypes (large Gram-positive rods), *Bacteriodes* spp morphotypes (small Gram-negative rods), Gram-variable bacillus, *Mobiluncus* spp. morphotypes (curved Gram-variable rods), curved Gram-negative rods, and Gram-positive cocci. A high score of 7–10 was based on the absence or only few *Lactobacilli* morphotypes and the more abundant presence of *Mobiluncus*.

Cytokine Panel

Cytokines were assayed in a multiplex format using an electrochemiluminescence platform and MS2400 imager for detection (Meso Scale Discovery, Gaithersburg, MD). The ultrasensitive kit had a lower limit of detection (LLOD) below 1 pg/mL with a wide dynamic range up to the ng/mL range. We focused on 3 proinflammatory cytokines of primary interest (IL-1 β , IL-6 and IL-8) and one regulatory cytokine (IL-2). Other cytokines with very low values or poor cross-reactivity were not analyzed. The absolute value (pg/mL) was determined as well as the concentration divided by CVF weight to consider the possible influence of specimen viscosity.

Isolation of DNA and rRNA Amplicon Sequencing

After thawing the vaginal swabs, DNA was isolated using the PowerSoil DNA isolation kit (MoBio, Carlsbad, CA). Purified DNA was quantified with a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and stored at -20°C in 10mM Tris buffer until sequenced. PCR amplification of the V4 variable region of the 16S rRNA gene using variable region specific primers (515F-806R) and amplicon sequencing were performed on an Illumina MiSeq by the Biosciences Division Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory (Argonne, IL). Sequences were analyzed using Quantitative Insights into Microbial Ecology (QIIME) [Caporaso et al., 2010a], de-multiplexed and quality filtered per QIIME defaults. Operational taxonomic units (OTUs) were assigned to the Greengenes (v. 2013_May) database [Caporaso et al., 2010b; DeSantis et al., 2006; McDonald et al., 2012; Werner et al., 2012] using PyNAST at 97% similarity through uclust and the closed reference OTU picking in QIIME. To limit errors when calculating the relative abundance due to differences in 16S rRNA gene copy number across bacterial taxa [Kembel et al., 2012], the OTU tables were normalized by dividing each OTU by the known or predicted 16S rRNA gene copy number using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [Langille et al., 2013]. Because the aim was to identify differences in the abundance of specific taxa, in addition to evaluating the microbial community structure, we followed the recommendations against rarefaction of 16S rRNA gene sequence data, and instead accounted for unequal sampling by normalizing the data through the parametric method, metagenomeSeq, implemented in QIIME [McMurdie and Holmes, 2014].

Faith's Whole Tree index of phylogenetic diversity was generated to examine the microbial richness of the specimen (i.e., alpha diversity) and weighted UNIFRAC dissimilarity matrices generated to assess differences in the community structure across individuals (i.e., beta diversity). Faith's Whole Tree index and Weighted UNIFRAC measures were chosen to account for both taxa abundance and phylogenetic relatedness [Lozupone and Knight, 2005; Lozupone et al., 2011]. Alpha and beta diversity analyses were both computed at the Operational Taxonomic Unit (OUT) level. Based on studies of the gut microbiome indicating that there is value in first conducting a Phylum level analysis, the ratio of *Firmicutes*-to-*Bacteroidetes* was considered as one factor that might distinguish the female's microbiome [Ley et al., 2006; Mariat et al., 2009]. Previous studies on the reproductive tract of female baboons showed that they tend to have a lower abundance of *Firmicutes* as compared to healthy women [Rivera et al., 2010]. All subsequent taxonomic analyses were based on genus level classifications. PICRUSt was also used to predict microbial metagenomes from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs in order to evaluate whether individual differences in the relative abundance of *Firmicutes* and *Bacteroidetes* would affect functional pathways involved in metabolic activity.

Reproductive Success

At our facility, adult female monkeys are typically bred with a timed-mating protocol after their first pregnancy. The cycling female is housed with an adult male for only 4–7 days at mid-cycle. Thus, it was possible to quantify the number of mating sessions required for each female to conceive. Because all pregnancies were monitored and births confirmed, parity

and gestational outcomes could be determined by the number of live births. The number of viable births by the time a female reached 10 years of age was employed as an index of reproductive success because it provided a metric of repeat conceptions in these multiparous females. In addition, 10 years was a modal age attained by most of the females evaluated.. Fifteen females from Study 1 were included this analysis (7 with low and 8 with high Nugent scores), as well as all 8 females from Study 2. Premature births did not occur, and all births were from unassisted vaginal deliveries at term.

Data Analysis

Following an evaluation of the descriptive statistics, analysis of variance with planned contrasts was used in Study 1 to compare females with Nugent scores >7 and those with scores below 6. Study 2 considered the influence of the *Firmicutes/Bacteroidetes* ratio and the relative abundance of *Lactobacilli* on other taxa, microbial richness and community structure. The alpha diversity index and the beta diversity distance matrix were generated using QIIME and the PCoA data and plots were generated with Paleontological Statistics (PAST) [Hammer et al., 2001]. Taxonomic composition was compared in QIIME with the nonparametric Kruskal-Wallis test and correlations with alpha and beta diversity evaluated with the Spearman nonparametric test and PERMANOVA, respectively. PERMANOVA is a permutation method used to assess differences in distance measures and assumes homogeneity of dispersion. Homogeneity in the distance matrices was verified by PERMDISP [Anderson, 2001]. Significance was set at $p < 0.05$. Values in tables and figures are presented as the mean $\pm$ standard error of the mean (SEM).

RESULTS

Nugent scores and CVF acidity

The Gram stain results are presented in Table 1. Large gram-positive rods, the morphotype of *Lactobacilli*, were not prominent in any females, but were observed in 11 of the 27 assessed monkeys. Based on Nugent's criteria, 8 of 27 monkeys (30%) were classified as having normal profiles, 8 were borderline (30%), and 11 (40%) would have met clinical criteria for BV. Subsequent analyses were based on a categorical division of Nugent scores as either low or high: below 6 or between 7–10, respectively. As predicted, acidity was related to Nugent scores. The pH of the CVF was more basic in females with a high Nugent score >7 (pH = 6.41 ± 0.26 for high Nugent score vs. 5.64 ± 0.19 for specimens with a lower Nugent score; $p < 0.05$). The Nugent scores were not associated with a female's age (Nugent <6 = mean 11.24 ± 1.23 years of age versus Nugent >7 = mean 11.91 ± 1.64 years of age).

Cytokines in CVF

The proinflammatory cytokine present at the highest concentration in the CVF was IL-1 β , significantly above the levels of IL-6 and IL-8 ($F[2,46] = 19.3$, $p < 0.001$) (Figure 1). Although secreted at lower levels, IL-8 was positively and significantly correlated with IL-1 β ($r = 0.40$, $p < 0.04$). The 3 proinflammatory cytokines tended to be higher, and IL-2 was lower, in females with elevated Nugent scores, but these differences did not reach statistical significance. Correcting for specimen viscosity did not alter these conclusions; IL-1 β remained the most prominent cytokine in the CVF.

Phylum level analysis

As shown in Figure 2A, analyses at the Phylum level indicated the *Firmicutes/Bacteroidetes* ratio was low as compared to the typical levels in women and in keeping with prior microbial assessments of the reproductive tract of female baboons [Rivera et al., 2010]. When individual variation was considered, the microbiota of the 4 females with a higher *Firmicutes/Bacteroidetes* ratio exhibited significantly more richness and a different community structure than observed in the 4 females with lower ratios ($p < 0.05$) (Figure 2B and 2C). KEGG Orthologs database was referenced to predict the potential influence on functional pathways, based on the estimated genomic content represented by the microbiota in each monkey. These predictions indicated that the differences in richness and community structure would be accompanied by significant shifts in many metabolic pathways, including carbohydrate, lipid and energy metabolism, xenobiotic biodegradation and metabolism, as well as a number of biochemical pathways implicated in human disease (Table 2).

Lactobacilli and Microbial Community Structure

Based on the 16S rRNA gene amplicon sequencing, *Lactobacilli* were present in the specimens from all 8 females, but the relative abundance was lower than typically found in women (Figure 3). The values ranged from 3.16 to 17.32% (mean = 7.05%). Eleven other genera were also present at abundances above 1%, with *Prevotella* spp being the most common (mean = 7%). The microbial profiles of the females were dichotomized into two subtypes based on a median split: either Low or High *Lactobacilli* (<5% or >6%). Alpha diversity tended to be greater in females with less *Lactobacilli*, but this trend did not reach statistical significance. In addition, it was attributable primarily to rare taxa and sequences that could not be identified at the genus level (Figure 3, Supplemental Figure 1).

Reproductive Success

The potential influence of elevated Nugent scores, the *Firmicutes/Bacteroidetes* ratio, and a low *Lactobacilli* abundance, on reproductive success was assessed by examining the number of pregnancies and births for the females assessed in Study 1 and 2 (Table 3). Females with high Nugent scores did not require significantly more mating sessions to conceive than did females with a low Nugent score, nor did they have fewer offspring by 10 years of age. Similarly, a low *Firmicutes/Bacteroidetes* ratio and less *Lactobacilli* did not undermine the rapidity with which a female conceived or reduce the number of infants birthed between 5–10 years of age. There was no difference in delivery mode, because there were no premature births, and all infants were from unassisted vaginal deliveries at term.

DISCUSSION

These findings are consistent with prior research on monkeys showing that the microbiota in their lower reproductive tract are diverse, even when living in the more hygienic conditions of a laboratory setting [Gravett et al., 2012; Yildirim et al., 2014]. The microbial profile is characterized by relatively low levels of *Lactobacilli*, and high numbers of Gram-negative bacteria, and thus superficially resembles the dysbiosis of women with BV [Ravel et al., 2011]. We generated both traditional Nugent scores and used culture-independent 16S rRNA gene sequencing, verifying the conclusions of others who have employed culture-based or

DNA-based techniques [Spear et al., 2012]. An early paper on the vaginal microbiota of 37 rhesus macaques was unable to isolate *Lactobacilli* in culture, and the investigators reported that they did not observe large Gram-positive rods, the morphotype indicative of *Lactobacillus* [Doyle et al, 1991]. We found that 16 of 27 monkeys had either none or few large Gram-positive morphotypes. A more recent article utilizing DNA-based sequencing reported that only 4 of 11 rhesus macaques had *Lactobacillus* sequences [Spear et al. 2010]. Another study employing 16S rRNA sequencing found that *Lactobacillus* represented less than 2% of sequences in the vaginal specimens from 16 rhesus monkeys (Hu et al., 2015). These results are in marked contrast to the healthy profile for women, in whom *Lactobacilli* are typically the dominant taxa [Ravel et al., 2011].

One exception to the general consensus that *Lactobacilli* are uncommon in the reproductive tract of nonhuman primates assessed rhesus monkeys of Chinese origin [Yu et al., 2009]. That paper reported many were colonized with *Lactobacillus johnsonii*, in contrast to the low abundance of *Lactobacilli* that we, and others, have found for rhesus monkeys derived originally from India [Spear et al., 2010]. Some researchers have taken advantage of this bacterial attribute of the monkey reproductive tract to investigate clinical treatments for changing the commensal bacteria [Scorpio et al., 2008]. Even in female rhesus monkeys, topical introduction of a sucrose gel vaginally for just 5 days markedly increased *Lactobacilli*, and decreased *Porphyromonas*, *Sneathia*, and *Mobiluncus*, and changed the acidity of their CVF [Hu et al., 2015]. It has also been possible to colonize the reproductive tract of rhesus monkeys with recombinant *Lactobacillus jensenii* expressing an antiviral protein, which afforded protection against retroviral infection [Lagenaur et al., 2011]. Thus, either providing a prebiotic energy source favorable to *Lactobacilli* or employing this taxon as a probiotic regimen appears to be sufficient to modify the polymicrobial diversity typically found in the vaginal milieu of female monkeys.

Our finding that 12 bacterial genera were present at >1% concurs with Spear et al. [2010] who reported that 9 taxa were usually present at >1%. Across the 11 monkeys in their study, 38 different taxa were present at >1%. There is also variation in which taxa are dominant across different facilities housing rhesus monkeys, with some researchers finding *Sneathia* and *Peptoniphilus*, and others reporting *Mobiluncus* and *Porphyromonas* to be more common. In our animals, *Prevotella* and *Peptoniphilus* were among the more abundant taxa. Females with relatively low *Lactobacilli* tended to have more alpha diversity in their community structure, but this trend did not reach statistical significance. The *Firmicutes*/*Bacteroidetes* ratio proved to be more informative with respect to understanding individual differences in the alpha and beta diversity measures. In addition, while this ratio and the relative abundance of *Firmicutes* were still much lower than in healthy women [Ling et al., 2010], KEGG predictions of metabolic function indicated that more *Firmicutes* would be associated with an increase in the energetic and metabolic activity of the commensal microbiota in the reproductive tract. Here it should be noted that whereas *Lactobacilli* are the primary contributor to the *Firmicutes* phylum in women [Fredicks et al., 2007], *Clostridia* are a major taxa in the vaginal microbiota of monkeys. Conversely, *Prevotella* are classified in a different Phylum, *Bacteroidetes*.

Neither a high Nugent score nor a low *Lactobacilli* abundance appeared to result in an exacerbated inflammatory condition, which concurs with previous evaluations of female baboons [Uchihashi et al., 2015]. This conclusion differs from one paper that indicated many female macaques do show signs of inflammation in the reproductive tract, even if it is not always due to more diverse microbiota [Spear et al., 2012]. IL-1 β was present at particularly high levels in all specimens, which is similar to findings on IL-1 β in the CVF from women. One paper on the CVF of monkeys also found that IL-8 levels were high, but clinical research on IL-8 as a sensitive and specific indicator of BV in women has been inconsistent [Cauci et al., 2003]. A close reading of papers on monkey CVF will also reveal significant differences in the reported concentrations for different cytokines, which likely reflects variation in the procedural steps used to collect and dilute the viscous matrix of the CVF, as well as differences in the specific binding of the assay antibodies to nonhuman primate cytokines.

Within any specific study, however, the cytokine levels in the CVF and the microbial profiles of monkeys have been reported to be fairly stable over time, even when specimens were obtained 8 months apart [Spear et al., 2012]. Some investigators did find variation associated with the menstrual phase and menses [Yu et al., 2009; Uchihashi et al., 2015], which is why we chose to collect specimens during the follicular phase for Study 1, and scheduled to avoid the time of menstrual bleeding. It is also known that recent sexual activity can influence both the microbiota and the acidity of the vaginal milieu. Because of the standardized breeding strategy used at our facility, and the fact that the females were not living with males, we were able to exclude the influence of recent sexual activity. But both in monkeys and humans, there is also evidence that promiscuity can affect the microbiota present in the reproductive tract. Specifically, having multiple sexual partners is a known risk factor for BV in women [Koumans et al., 2007]. In addition, physical orientation during sexual intercourse, which in monkeys is always with the male posterior, may result in some ano-genital transfer of gut microbiota, especially for *Prevotella* that are particularly abundant in the intestines and stool of monkeys [Zozaya et al., 2016; Amaral et al., 2017]. However, this microbial diversity and the low *Lactobacilli* did not affect the female monkeys' reproductive efficiency and success over time. Low *Lactobacilli* in the reproductive tract appears to be the natural condition for most female primates other than women. The absence of an influence low *Lactobacilli* and high microbial diversity on reproductive efficiency, miscarriage, and full-term delivery differs from humans [Leitich and Kiss, 2007]. BV in women has also been associated with an increased risk for sexually transmitted infections and secondary infertility, in addition to premature births [Jones et al., 2010; Kenyon et al., 2013].

Several limitations of this research should be acknowledged. In particular, the evaluation of reproductive success was based on a retrospective analysis of conceptions and births in middle-aged monkeys after their microbiota were assessed from a single specimen in adulthood. Although there is evidence for stability in the microbial profiles over time, it is known that the taxa can change with reproductive phase, including pregnancy, and potentially with age. A female's age was not associated with the likelihood of a high Nugent score in our survey, but we did exclude old age and thus menopausal animals. It should also be acknowledged that the number of monkeys assessed was relatively small. But given that

our Nugent scoring and microbiome assessments were confirmatory of the extant literature, we are confident of the general conclusions. A longitudinal and prospective assessment is still warranted to verify that there is no influence of the vaginal microbiota on reproductive success.

In sum, the current research confirms that female monkeys have a more heterogeneous microbial community in their lower reproductive tract than typically seen in women. *Lactobacilli* are not as abundant, and thus the vaginal milieu is also less acidic. We did not see signs of the negative sequelae known to occur in women, including an increased risk for sexually transmitted infections, pelvic inflammatory disease, and preterm labor [Kaul et al., 2007; Nagot et al., 2007; Jones et al., 2010]. It is important to learn more about microbial-host interactions in the reproductive tract and to determine if the females of nonhuman primate species can provide further insight into how the commensal bacteria contribute to reproductive health. Similar to observations in women, experimental infections in female monkeys have provided strong evidence that a greater bacterial diversity may increase the susceptibility to viral pathogens, such as simian immunodeficiency virus and Herpes viruses. The vaginal microbiota of female primates also more closely resembles the profiles of prepubertal and postmenopausal women, which is characterized by lower *Lactobacilli* and pH (Ravel et al., 2011). However, relatively little is still known about how the composition of vaginal microflora confers resilience at either low or high bacterial diversity. Further research is needed to understand how the gravid female monkey can tolerate microbial diversity as well as the high cytokine levels in her CVF, given that administering endotoxin or a proinflammatory cytokine like TNF-alpha can result in miscarriage and premature delivery even in monkeys [Adams et al., 2011].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Three proinflammatory mediators (IL-1 β , IL-6, and IL-8) and one regulatory cytokine (IL-2) in the cervicovaginal fluid (CVF) of monkeys, comparing specimens from females with High Nugent (7–10) and Low Nugent scores (< 6). IL-1 β was present at significantly higher levels than the other assayed cytokines, but was not indicative of an exacerbated inflammatory state in females with higher Nugent scores. IL-8 concentrations were lower but positively correlated with IL-1 β ($p < 0.05$).

Figure 2.

Phylum level analysis of the vaginal microbiota in 8 female rhesus monkeys focusing on Firmicutes/Bacteroidetes ratio. A. The Firmicutes/Bacteroidetes ratios were relatively low compared to women, but distinguishable individual variation was evident when a median split was used to categorize females with Lower or Higher ratios (<3.4 versus >3.5). B. Ratios indicative of relatively more Firmicutes were significantly correlated with more alpha diversity, $p < 0.03$ (richness of the microbiome). At the Class level, Clostridia were a major component of the Firmicutes. C. Principal coordinates analysis (PCoA) showed that the beta diversity values were also significantly different between females with high and low F/B ratios ($p < 0.05$).

Figure 3.

16S rRNA gene amplicon sequencing confirmed reports of a diverse polymicrobial community structure in the lower reproductive tract of female monkeys. A. Lactobacilli were not predominant, ranging from 3–17%, and 11 other taxa were present at abundances above 1%. B. Further categorization on the basis of relative abundance of Lactobacilli (<4.9 or >5%) indicated only a non-significant trend for less alpha diversity in the females with the most Lactobacilli. C. Beta diversity values were not affected by the relative abundance of Lactobacilli.

TABLE 1

Gram stain results for 27 non-gravid adult female rhesus macaques. The values are the number of females (and percent) with vaginal smears evincing each bacterial morphotype.

Type	None-Rare	Few-to-Many Observed
Large Gram-positive rods *	16 (59%)	11 (41%)
Small Gram-positive rods	13 (48%)	14 (52%)
Curved Gram-negative rods	15 (56%)	12 (44%)
Curved Gram-variable rods	22 (85%)	5 (15%)
Small Gram-negative rods	13 (48%)	14 (52%)
Gram-variable <i>bacilli</i>	19 (70%)	8 (30%)
Gram-positive <i>cocci</i>	5 (19%)	22 (81%)

* indicative of *Lactobacilli* morphotype

TABLE 2

Significant differences predicted for metabolic pathways associated with the microbiota of female monkeys when there was a higher *Firmicutes/Bacteroidetes* ratio and more alpha and beta diversity in the lower reproductive tract.

KEGG Prediction	Test Statistic	P	FDR-P	Low FtoB	High FtoB	Difference
Amino Acid Metabolism	5.333	0.021	0.025	146651.75	280313.25	133661.50
Carbohydrate Metabolism	5.333	0.021	0.025	166650.00	317946.25	151296.25
Lipid Metabolism	5.333	0.021	0.025	44285.50	83869.75	39584.25
Energy Metabolism	5.333	0.021	0.025	94090.25	177064.00	82973.75
Biosynthesis of Other						
Secondary Metabolites	5.333	0.021	0.025	13130.75	24993.50	11862.75
Cell Growth and Death	5.333	0.021	0.025	9139.50	17094.25	7954.75
Cell Motility	5.333	0.021	0.025	29040.75	72608.75	43568.00
Cell Processes, Signaling	5.333	0.021	0.025	62001.25	115836.50	53835.25
Environmental Adaptation	5.333	0.021	0.025	2605.25	5153.00	2547.75
Enzyme Families	5.333	0.021	0.025	36298.50	68239.50	31941.00
Folding, Sorting, Degrade	5.333	0.021	0.025	42237.50	76648.25	34410.75
Genetic Info Processing	5.333	0.021	0.025	47384.75	86834.50	39449.75
Glycan synthesis/metab	5.333	0.021	0.025	39439.00	64445.75	25006.75
Membrane Transport	5.333	0.021	0.025	203556.00	398436.50	194880.50
Metabolism	5.333	0.021	0.025	40562.50	73967.25	33404.75
Cofactors/Vitamin Metab	5.333	0.021	0.025	70369.25	128956.00	58586.75
Other Amino Acid Metab	5.333	0.021	0.025	25049.75	44651.25	19601.50
Metabolism of Terpenoids and Polyketides	5.333	0.021	0.025	28167.50	51440.25	23272.75
Nucleotide Metabolism	5.333	0.021	0.025	74589.00	135249.75	60660.75
Replication and Repair	5.333	0.021	0.025	158817.50	295399.00	136581.50
Signal Transduction	5.333	0.021	0.025	23567.50	47747.50	24180.00
Signaling Molecules	5.333	0.021	0.025	3469.25	5518.25	2049.00
Transcription	5.333	0.021	0.025	44184.00	88610.00	44426.00
Translation	5.333	0.021	0.025	106552.75	196988.25	90435.50
Transport and Catabolism	5.333	0.021	0.025	4400.25	7341.50	2941.25
Xenobiotic Biodegradation and Metabolism	5.333	0.021	0.025	27286.75	51939.75	24653.00

<u>KEGG Prediction</u>	<u>Test Statistic</u>	<u>P</u>	<u>FDR-P</u>	<u>Low FtoB</u>	<u>High FtoB</u>	<u>Difference</u>
Immune System	5.333	0.021	0.025	1445.50	2600.00	1154.50
Nervous System	5.333	0.021	0.025	1561.50	2990.25	1428.75
Endocrine System	5.333	0.021	0.025	4649.75	8735.50	4085.75
Excretory System	5.333	0.021	0.025	383.25	573.00	189.75
Cardiovascular Diseases	4.984	0.026	0.029	1.00	8.00	7.00
Immune System Diseases	5.398	0.020	0.025	885.75	1465.25	579.50
Cancers	5.333	0.021	0.025	2006.25	3386.50	1380.25
Infectious Diseases	5.333	0.021	0.025	6550.50	11711.00	5160.50
Metabolic Diseases	5.333	0.021	0.025	1876.50	3322.50	1446.00

TABLE 3

The reproductive success of female rhesus monkeys was not affected by high Nugent scores, low *Firmicutes/Bacteroidetes* ratios, or the low abundance of *Lactobacilli* in the lower reproductive tract.

Designation	Infants by 10 years *	Mating sessions **
Low Nugent (<6) ***	3.3 (.4)	1.8 (.2)
High Nugent (7–10)	3.6 (.4)	2.2 (.4)
Low Firmicutes/Bacteroidetes ****	4.3 (.3)	1.8 (.4)
High Firmicutes/Bacteroidetes (>3.5)	3.3 (.5)	2.3 (.2)
Low Lactobacilli (<4%)	4.3 (.3)	1.8 (.3)
High Lactobacilli (5–17%)	3.0 (.6)	2.3 (.3)

* Total number of infants birthed by 10 years of age provided a measure of fecundity.

** Number of mating sessions with a breeder male needed to conceive was used as an index of fertility.

**** Reproductive histories of 15 females in Study 1 were compared (7 Low and 8 High Nugent scores), and for all 8 females in Study 2 (4 Low and 4 High F/B ratios and similarly 4 with Lower and 4 Higher relative *Lactobacilli* abundance).

**** Categorization of the *Firmicutes/Bacteroidetes* ratio and *Lactobacilli* was done with the same monkeys, but the ranking resulted in two being assigned to different subgroups.