RESEARCH ARTICLE

Influence of Age, Reproductive Cycling Status, and Menstruation on the Vaginal Microbiome in Baboons (*Papio anubis*)

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The vaginal microbiome is believed to influence host health by providing protection from pathogens and influencing reproductive outcomes such as fertility and gestational length. In humans, age-associated declines in diversity of the vaginal microbiome occur in puberty and persist into adulthood. Additionally, menstruation has been associated with decreased microbial community stability. Adult female baboons, like other non-human primates (NHPs), have a different and highly diverse vaginal microbiome compared to that of humans, which is most commonly dominated by *Lactobacillus* spp. We evaluated the influence of age, reproductive cycling status (cycling vs. non-cycling) and menstruation on the vaginal microbiome of 38 wild-caught, captive female olive baboons (*Papio anubis*) by culture-independent sequencing of the V3–V5 region of the bacterial 16S rRNA gene. All baboons had highly diverse vaginal microbial communities. Adult baboons had significantly lower microbial diversity in comparison to subadult baboons, which was attributable to decreased relative abundance of minor taxa. No significant differences were detected based on cycling state or menstruation. Predictive metagenomic analysis showed uniformity in relative abundance of metabolic pathways regardless of age, cycle stage, or menstruation, indicating conservation of microbial community functions. This study suggests that selection of an optimal vaginal microbial community occurs at puberty. Since decreased diversity occurs in both baboons and humans at puberty, this may reflect a general strategy for selection of adult vaginal microbial communities. Comparative evaluation of vaginal microbial community development and composition may elucidate mechanisms of community formation and function that are conserved across host species or across microbial community types. These findings have implications for host health, evolutionary biology, and microbe-host ecosystems. Am. J. Primatol. 77:563–578, 2015.   © 2015 Wiley Periodicals, Inc.

Key words: non-human primates; baboon; microbiome; vagina; bacterial 16S rRNA gene

INTRODUCTION

The term "microbiome," in the context of host–microbial interactions, refers to the microbial community at a particular body site in combination with the genetic information encoded by this community [Cho & Blaser, 2012]. The normal mucosal microbiome of humans and animals is believed to benefit host health through protection from pathogens and contribution to critical host functions including immune education, metabolism, and epithelial development [Ding & Schloss, 2014; Lozupone et al., 2012; Ravel et al., 2013; Sartor & Mazmanian, 2012]. The study of mucosal microbial communities has undergone a revolution over the past 10–15 years with the implementation of culture-independent, high throughput sequencing methods [Foster et al., 2012]. These DNA sequence-based techniques generate a more complete picture

of microbial communities than was possible with culture-based methods, since the majority of all bacterial species identifiable by DNA-based techniques are non-cultivable [Foster et al., 2012].

The vaginal microbiome represents a mucosal microbial community that is subject to frequent environmental perturbations due to pathogen exposure and physiologic fluctuations inherent to the reproductive cycle [Farage et al., 2010; Gajer et al., 2012; Hickey et al., 2012; Zhou et al., 2004]. A "microbial community" consists of the composition of species in an ecologically grouped, multispecies assemblage of bacteria, considered with respect to taxonomy and relative abundance [Konopka, 2009]. The "normal" vaginal microbial community of healthy women is classically defined by a predominance of *Lactobacillus* spp. *Lactobacillus* ferment glycogen into lactic acid, generating a low vaginal pH that is thought to prevent colonization and proliferation of potentially pathogenic organisms [Hickey et al., 2012; Linhares et al., 2011; O'Hanlon et al., 2011]. This community structure is not, however, universal to all primates. Although female non-human primates (NHPs) undergo similar reproductive physiological changes, they have a higher vaginal pH and significantly different vaginal microbial community composition than women [Hashway et al., 2014; Rivera et al., 2010,2011; Spear et al., 2010, 2012; Stumpf et al., 2013; Yildirim et al., 2014]. Specifically, the NHP vaginal microbiome has much greater diversity and a striking paucity of lactobacilli in comparison to that of women. Host-species was recently shown to be the most significant factor influencing vaginal microbial composition in a comparative analysis of humans and eight species of NHPs, yet inter-species host microbial community differences are not entirely explained by host phylogenetic differences [Rivera et al., 2010; Yildirim et al., 2014]. Furthermore, there is inter-individual variation within host species. In humans, approximately 27% of healthy, asymptomatic adult women have vaginal microbial communities that are not *Lactobacillus* dominant, and are highly diverse [Ravel et al., 2011]. For women with a *Lactobacillus* dominant vaginal microbiome, different species of *Lactobacillus* may predominate [Ravel et al., 2011]. In baboons, a recent study on a small number of baboons evaluated over 6 months showed that inter-animal variation exceeded intra-animal variation over the course of the study [Hashway et al., 2014].

With respect to specific bacterial composition at the phylum level, Firmicutes predominate in both the human and baboon adult vaginal microbiome, yet the species-level composition of this phylum differs in these hosts [Hashway et al., 2014; Rivera et al., 2010, 2011; Stumpf et al., 2013; Yildirim et al., 2014]. In the baboon, the vaginal Firmicutes consist of a diverse array of the anaerobic, polyphyletic class Clostridia,

while in the majority of humans, vaginal Firmicutes are comprised almost entirely of the genus *Lactobacillus*, within the class Bacilli [Hashway et al., 2014; Rivera et al., 2010, 2011; Spear et al., 2010, 2012; Stumpf et al., 2013; Yildirim et al., 2014]. In addition, the baboon vaginal microbiome has a greater representation of other phyla, including Fusobacteria (a phylum that includes many species pathogenic in humans), Bacteroidetes, Proteobacteria, Actinobacteria, and others that are minimally represented in the majority of adult women [Hashway et al., 2014; Rivera et al., 2010, 2011; Spear et al., 2010, 2012; Stumpf et al., 2013; Yildirim et al., 2014]. Evolutionary or ecological factors influencing the establishment of a higher diversity vaginal microbiome in the baboon and other NHP species are not known, nor is it known whether this community changes over the lifetime of the animals.

Both baboons and humans must contend with greater potential for pathogen exposure with post-pubertal onset of sexual activity. In women, age-related changes in the vaginal microbiome are correlated with levels of estrogen [Farage et al., 2010; Hickey et al., 2012]. In young children, the vaginal microbiome consists of a diverse mixture of aerobes, strict anaerobes, and enteric organisms [Hammerschlag et al., 1978; Hickey et al., 2012]. This changes during adolescence to the lower diversity, *Lactobacillus*-predominant community of adulthood [Gajer et al., 2012; Hammerschlag et al., 1978; Lamont et al., 2011; Ravel et al., 2011; Zhou et al., 2004]. Community shifts and decreased stability have been correlated with menstruation in humans [Gajer et al., 2012; Hickey et al., 2013; Lopes dos Santos Santiago et al., 2011]. Additionally, follicular phases (higher estradiol) of the menstrual cycle have been associated with higher levels of community stability [Gajer et al., 2012]. The effects of age or reproductive cycle–associated microbial changes in NHPs have not been extensively investigated. In particular, it is unknown whether the age and cycle-dependent alterations seen in humans are unique. Evaluation of the NHP vaginal microbiome in relation to life cycle or reproductive cycle stages may provide comparative insight into strategies used by host-microbial ecosystems to cope with the hormonal alterations and increased potential pathogen exposure associated with sexual maturity.

In this study, we evaluated the vaginal microbiome of wild-caught, captive olive baboons (*Papio anubis*) with respect to age and reproductive cycle phase. Olive baboons share many anatomic and physiologic reproductive features with humans, including non-seasonal sexual receptivity and a relatively straight cervical canal, in contrast to the seasonal sexual receptivity and tortuous cervical canal of the rhesus macaque (*Macaca mulatta*) [Fox, 2002; VandeBerg et al., 2008]. These features may lead to increased potential exposure to pathogens. To

our knowledge, alterations of the baboon vaginal microbiome with respect to age or reproductive cycle have not been evaluated. The specific objective of this study was to characterize differences in the vaginal microbial community of wild-caught, captive baboons based on age, reproductive cycle stage, and menstruation. Based on human studies, we hypothesized that adult baboons would have decreased microbial diversity, potentially due to selection for the optimal mixture of bacteria necessary for host health during reproductive life.

METHODS

Humane Care Guidelines and Regulatory Oversight

All aspects of live animal work in this study were approved by the Institutional Review Committee (IRC) at the Institute of Primate Research (IPR) in Karen, Kenya (approval number IRC/03/12). The IRC policies for humane animal care and use adhere to the 3R's [Russell & Burch, 1959], pertinent Kenyan law (Cap 360: Prevention of Cruelty to Animals Act), and the International Guiding Principles for Biomedical Research Involving Animals (CIOMS, ICLAS 2012). These principles are consistent with the Principles for the Ethical Treatment of Non-Human Primates (American Society of Primatology). This study received an off-site exemption from the University Committee for the Care and Use of Animals (UCUCA).

Study Site and Population

This study was conducted at the IPR, which was selected based upon our successful previous collaborations [Bell et al., 2011; Bergin et al., 2013] and the availability of a large population of wild-origin, genetically diverse baboons. Additionally, this institute has extensive expertise in the care and use of baboons, particularly in the context of reproductive disease models [Chai et al., 2007; D'Hooghe et al., 2004; Nyachieo et al., 2009]. IPR is a non-profit institution first established in 1960 and is a WHO collaborating center, an African ANDI Center of Excellence in Preclinical Research, an Associate Partner of the European Union Primate Network (EUPRIM), and has statutory compliance and registration with the NIH Office for Laboratory Animal Welfare (foreign institution assurance # A5796-01).

The study population initially consisted of 49 female olive baboons (*Papio anubis*). Animals were each sampled once during the period of July 17 and 18, 2012, coinciding with scheduled surveillance for tuberculosis (TB). TB testing is a routine screening procedure for NHPs, and all the baboons in this study tested negative for TB. The population represented a convenience sample of the available animals and was not pre-selected for age or reproductive cycle stage. The animals were uniquely identified by tattoos and are identified in this study as Baboon 1, 2, 3, …, 49. Additional metadata consisted of weight, estimated age, menstrual cycle stage, reproductive state, and physical and cervical examination findings.

Housing and Health Status

Animals in the main colony were housed in outdoor group enclosures consisting of one male and multiple females under ambient temperature and humidity. Protection from weather conditions and wildlife was provided by the enclosure roofing and natural bedding materials, and the wire-enclosure and perimeter fencing. The animals were fed monkey biscuits, and fresh fruits and vegetables. Animals in quarantine had been trapped from various sites in Kenya under permit from the Kenyan Wildlife Service. In brief, wild baboons reported to the Kenyan Wildlife Service as nuisance animals were trapped using baited live traps. Animals were transported to IPR and housed in quarantine for 90 days in small groups for acclimation, disease surveillance, and conditioning for endo and ectoparasites. The animals in this study were healthy without symptoms of infectious disease and none had been recently treated with antibiotics.

Sample Collection

Animals were sedated with an intramuscular injection of a mixture of 9.5 mg/kg ketamine (Rotex Medica, GMBH, Tritau, Germany) and 0.5 mg/kg xylazine (Rompun®, Bayer, Pittsburg, PA). Animals were weighed and underwent physical examination, including rectoabdominal bimanual uterine palpation, and cervical evaluation using a vaginal speculum. None of the animals had signs of reproductive or systemic diseases. With the animal in ventral recumbency, vaginal swab samples (Copan 307C, UTM™ minitip flocked swabs, Copan Diagnostic, Inc., Murrieta, CA) were taken by rolling the dry swab on the sides and dorsal wall of the distal vagina (approx. 2.5–5 cm from the introitus). The swabs were placed in liquid Amies media and stored on ice for approximately 30 min to 1 hr, then moved to storage at −70°C. The samples were transported under controlled temperature conditions (dry ice) by a commercial sample shipper (World Courier, New Hyde Park, NY) to the University of Michigan for DNA extraction and analysis.

Metadata and Subgroup Identification

Animals were categorized into adults or subadults based on body weight and physical appearance. Overall, body weights for adults were higher than subadults; 80% of subadults weighed less than

11 kg, which was the lowest weight for any of the adults included in this study (weight range: subadults = 9.2–13.5 kg, adults = 11–19 kg). Additionally, animals were characterized as adults if they had evidence of previous pregnancy (based on cervical morphology) or lactation (presence of elongated nipples [Altmann et al., 1981]). One animal could not be accurately classified as adult or subadult and was excluded from the age comparison.

Reproductive cycle phase was assessed based on perivulvar swelling at the time of the examination by an experienced observer using a modification of an established system [VandeBerg et al., 2008]. In brief, non-cycling animals had flat perivulvar and perianal skin (stage 0). Animals in the pre-ovulatory (follicular) phase of the menstrual cycle had increasing perivulvar swelling and turgidity (stage 1–3) reaching maximal swelling at the time of ovulation (stage 4). The luteal (secretory) phase of the cycle was represented by decreasing turgidity of the perivulvar skin (stage 5–6) culminating in menstruation (stage 7) with evidence of blood. Pregnancy (stage 8) was determined by abdominal or rectoabdominal palpation [Tardif et al., 2012].

For subgroup comparison based on age, 23 of the 38 total animals for which sequence was available were utilized (Table I). These represented 14 reproductively mature, non-pregnant adult, and nine

subadult (peripubertal) animals. The remaining animals were excluded due to pregnancy ($N = 3$), lactation ($N = 10$), old age (menopausal, $N = 1$) or inability to estimate age ($N = 1$). For subgroup comparison based on reproductive cycle, 31 of the 38 total animals were utilized (Table I). These represented 12 cycling (stages 1–7) and 19 non-cycling (stage 0) animals. The remaining animals were excluded due to pregnancy ($N = 3$), unknown cycle stage ($N = 3$) or old age (menopausal, $N = 1$). For comparison based on menstruation, 12 of the total 38 animals were utilized (Table I). These represented three menstruating (stage 7) and nine non-menstruating (stage 1–6) animals. The remaining animals were excluded due to pregnancy ($N = 3$), unknown cycle stage ($N = 3$), old age ($N = 1$) or non-cycling state (stage 0, $N = 19$).

DNA Extraction

Extraction of DNA from vaginal swab tips was performed with the Biomek ®FX^P (Beckman Coulter, Inc., Indianapolis, IN), a laboratory automated work station to optimize the accuracy and the efficiency of the isolation process. A Mo Bio PowerSoil®- htp 96 Well Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) was used due to its previously demonstrated suitability for vaginal microbial samples [Hashway et al., 2014] and high purity of the isolated DNA (http://www.mobio.com/).

Amplification and Sequencing of 16S rRNA Genes

Amplification of a 660 bp fragment of the hypervariable V3–V5 region of the 16S rRNA gene was performed using primer A (adapter A+ barcode + 926R) and primer B (adapter B+ 357F) according to the protocol from the Human Microbiome Project (HMP) Consortium (http://www. hmpdacc.org/doc/16S_Sequencing_SOP_4.2.2 pdf) and as previously described [Hashway et al., 2014] with modifications as follows. To maximize the amount of specifically amplified DNA, a touchdown PCR strategy [Don et al., 1991; Hecker & Roux, 1996; Korbie & Mattick, 2008] and more cycles (total 40 cycles) were used. One micro liter of extracted DNA and 0.2 μM each of primer A and primer B were used under the following thermal cycler conditions: 95°C for 2 min; 20 cycles of 95°C (20 sec), annealing temperatures starting at 60°C (30 sec) and decreasing by 0.5°C per cycle until reaching 50°C, followed by elongation at 72°C (5 min). This was followed by 20 additional cycles of 95°C (20 sec), 50°C (30 sec), and 72°C (5 min). The final product was held at 4°C. Sample purification and library construction were performed as previously described [Hashway et al., 2014]. Sequencing was performed using the Roche

TABLE I. Characteristics of the 38 Wild-Caught, Captive Baboons

Category	Number of animals (total $N = 38$)
Estimated age[a]	
Adults	27
Subadults	9
Menopausal	1
Unknown	1
Cycle stage	
Cycle 0 (non-cycling)	19
Cycle 1 (follicular phase)	2
Cycle 2 (follicular phase)	2
Cycle 3 (follicular phase)	0
Cycle 4 (ovulation)	2
Cycle 5 (luteal phase)	2
Cycle 6 (luteal phase)	1
Cycle 7 (menstruation)	3
Pregnant	3
Unknown	3
Menopausal	1
Lactating	10
Weight	
<11 kg	8
11–12.9 kg	8
13–14.9 kg	10
15–16.9 kg	6
≥17 kg	6

[a]See methods for description of age estimation.

454 GS FLX Titanium platform following the manufacturer's instructions (Roche 454 Life Sciences, Branford, CT).

Sequence Processing

All sequence processing and microbial community analyses were performed using commands within the software program mothur (version 1.31.2 and 1.33.3), according to the Schloss SOP as of October 2014 (http://www.mothur.org/wiki/454_SOP) [Schloss et al., 2009; Schloss et al., 2011]. Sequences with <200 bases, ambiguous bases, homopolymers >8 bases, or erroneous barcodes were removed. Sequences were aligned against those in a SILVA reference alignment using the Needleman-Wunsch and NAST algorithms [Pruesse et al., 2007; Schloss, 2009]. Sequences not sharing defined alignment space were removed. The UCHIME algorithm was used to identify chimeras [Edgar et al., 2011]. Sequences were classified using the Wang method (at an 80% confidence cutoff) with the Ribosomal Database Project (RDP) reference files (trainset9_032012.pds.fasta and trainset9_032012.pds.tax) [Wang et al., 2007]. Sequences classified as "Chloroplast", "Mitochondria" or "unknown kingdom" were removed. Sequences were then subsampled to normalize the numbers of sequences in each sample before data analysis.

Data Analysis

Microbial community analysis was undertaken using an operational taxonomic unit (OTU) approach. This approach involves grouping based on a priori sequence similarity, thus avoiding bias introduced by species assignation from sequence databases. Sequences were clustered into OTUs using a difference of 3% to define OTUs (approximately at the level of species differences). Microbial diversity is based upon species richness, defined as the number of OTUs present, and species evenness, defined as the proportion or relative abundance of different OTUs. Diversity was estimated by the Shannon diversity index, which is proportional to the natural logarithm of the relative abundance of each OTU [Magurran, 1988]. Microbial community richness was evaluated using the observed number of OTUs (S_{obs}), and the Chao1 richness estimator [Chao, 1984]. The latter is an estimate of expected richness in a given bacterial community and is predicted from the frequencies of rare OTUs present [Chao et al., 2009]. Evenness was determined by calculating the Shannon evenness score (Shannon equitability score) from the Shannon diversity index. Unpaired t tests (GraphPad Prism version 6.02 for Windows, GraphPad Software, San Diego, CA) were used to compare richness, evenness, and diversity using a 95% confidence interval. Unpaired t-tests

were also used to compare specific phylum-level relative abundances between groups. A Dirichlet Multinomial Mixtures (DMM) model [Holmes et al., 2012] was used to identify community types based on OTUs alone. OTU-based comparison was performed by construction of a distance matrix of the dissimilarity (1-similarity) between microbial communities and the Yue & Clayton measure of dissimilarity was calculated using the θ_{YC} calculator [Yue & Clayton, 2005]. The Yue Clayton measure is a comparison of community similarity that accounts for relative abundance of OTUs as well as community membership (the particular OTU or species). Results of the θ_{YC} dissimilarity calculation were visually represented by principal coordinates analysis (PCoA) and statistical significance was evaluated by analysis of molecular variance (AMOVA) [Anderson, 2001]. Significance of AMOVA was defined as $P < 0.05$, after Bonferroni correction for multiple comparisons. Phylogeny-based comparison of microbial communities was performed by weighted and unweighted UniFrac analysis [Lozupone & Knight, 2005; Lozupone et al., 2007]. Significance was defined as $P < 0.05$, after Bonferroni correction for multiple comparisons. Additionally, microbial community function was evaluated by predictive metagenome (microbial DNA) analysis using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). PICRUSt is a recently developed phylogeny-based computational tool that predicts the functional capacity of microbial communities by correlation of the species present to reference databases of microbial genomes [Langille et al., 2013]. OTUs were normalized by 16S rRNA copy number, and KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs (KOs) were predicted [Langille et al., 2013; Muto et al., 2013]. The information obtained from PICRUSt was further processed by HUMAaN (The HMP Unified Metabolic Analysis Network) to predict functional metabolic pathways [Abubucker et al., 2012]. Metabolicpathways (modules) were then grouped into larger categories using the KEGG module database (http://www.genome.jp/kegg/module.html) [Mutoet al., 2013].

RESULTS

Microbial Community Sequence Metrics: All Samples

Vaginal microbial swabs were obtained at a single time point from 49 wild-caught, captive olive baboons (*Papio anubis*). The characteristics of the study population are summarized in Table I and described in the methods. The structure of the vaginal microbial community for each animal was determined by sequencing the V3–V5 region of the 16S rRNA-encoding gene. Of the 49 vaginal samples, 11 yielded

insufficient DNA for sequence analysis, likely due to insufficient bacteria present in the original sample or deterioration of DNA in samples after freeze–thaw. After quality control, a total of 423,023 sequence reads were generated from the remaining 38 samples. The mean length of sequence was 256.8 bases (range 249–270, median 257). The complete data set was subsampled to 2,107 sequences per animal, representing the minimum number of sequences in any animal that passed quality control. Each sample yielded a mean of 42.1 ± 29.3 (mean $\pm$ SD) OTUs (median $= 35.7$, range 18.7–196.0), with sequence differences of 3% defining an OTU. Since the total number of unique OTUs across all baboons in the dataset was 781, each baboon vaginal microbial community represented a highly individualized subset within the potential species pool for this anatomic site. To determine whether the observed OTUs adequately reflected the true number of OTUs, we calculated the mean Chao1 estimate of richness, a rarefaction curve, and the average Good's coverage. The Chao1 estimate was 56.1 ± 41.3 (median $= 46.16$, range 22.4–255.0), indicating slight underrepresentation. The rarefaction curve (measure of sampling sufficiency) showed a plateau in the majority of samples at the level of subsampling ($\sim$2100 sequences), except for two outliers (baboon 1 and 2) (data not shown). Nevertheless, the average Good's coverage [Good, 1953] (where "perfect" coverage $= 1$) was 0.995 ± 0.005 (median $= 0.996$, range $= 0.974$–0.998), indicating that the true number of OTUs was adequately represented.

Microbial Community Descriptive Features: All Baboons

The bacterial phyla present at a relative abundance of $\geq 1\%$ in the animals in this study are shown in Figure 1A. The three most abundant phyla across all baboons were Bacteroidetes ($26.12 \pm 17.45\%$), Fusobacteria ($24.16 \pm 21.85\%$), and Firmicutes ($32.72 \pm 23.08\%$) (mean $\pm$ SD). Ten of 38 (26.3%) animals had >10% abundance of Tenericutes, Proteobacteria, or Spirochetes, which are phyla consisting of mostly anaerobic or facultative anaerobic bacteria [Brenner et al., 2005; Gupta et al., 2013; Whitman, 2010]. A variable number (range 0–35.8% per baboon) of sequences could not be assigned to a phylum and remained unclassified based on the sequence fragment available and the available reference database. Within Firmicutes, the polyphyletic class Clostridia ($76.9 \pm 23.3\%$, range 10.0–98.8%) predominated over the class Bacilli ($14.8 \pm 18.2\%$, range 0–65.4%), which includes the genus *Lactobacillus* (Fig. 1B).

Further descriptive analysis was undertaken at the genus level. A mean of 28.53 ± 10.28 (range 17–80) genera were present in each baboon. The bacterial genera present at a relative abundance of >1% in the animals in this study are shown in Figure 1C. The average number of genera present at >1% in any animal was 10.24 ± 4.04. The top 20 genera averaged across all animals are shown in Table II. *Lactobacilli* were present in only 16% of animals and at prevalence of $1.25 \pm 3.34\%$ (range 0–14.95%).

Baboon vaginal microbial communities were evaluated for a priori segregation into community types by attempting to identify community types using Dirichlet Multinomial Mixtures (DMM) [Holmes et al., 2012]. For DMM, there was no statistically significant segregation into distinct community types (data not shown). A θ_{YC} distance matrix was generated and visualized using principal coordinates analysis (PCoA). The first two of 36 PCoA (Principal Coordinates Analysis) axes were plotted (Fig. 2A), which represent 20.4% (axis 1) and 8.6% (axis 2) of the data variance. Since no significant community types could be identified by DMM, no significant *a priori* segregation into community types is present.

Community Analysis within Subgroups: Age, Reproductive Cycle, Menstruation

We next performed further evaluation for segregation into community types between predefined subgroups of animals (see Methods Section and Table I). Specifically, vaginal microbial communities were compared between reproductively mature, non-pregnant, non-lactating adults ($N = 14$) and subadults (peripubertal, $N = 9$), between cycling (stages 1–7, $N = 12$) and non-cycling or resting phase (stage 0, $N = 19$) animals, and between menstruating (stage 7, $N = 3$) and cycling but non-menstruating (stages 1–6, $N = 9$) animals (Fig. 3).

Community Analysis Within Subgroups: Membership and Community Structure

Vaginal microbial community structures within each subgroup (age, reproductive cycle, menstruation) were compared by calculation of the OTU-based θ_{YC} dissimilarity measure (Table III). There were no significant differences in community structures within any of the subgroups. θ_{YC} dissimilarity was visualized via PCoA plots for each subgroup, which showed that animals did not cluster based on age, cycle stage, or menstruation (Fig. 2B–D). Additionally, community structures were compared via weighted and unweighted UniFrac (phylogeny-based analysis). No significance was detected using unweighted UniFrac analysis (Table III), yet significant differences were detected in all groups examined using weighted UniFrac analysis. Weighted UniFrac and Yue Clayton dissimilarity index emphasize relative abundance of taxa [Chen et al., 2012], while unweighted UniFrac emphasizes membership.
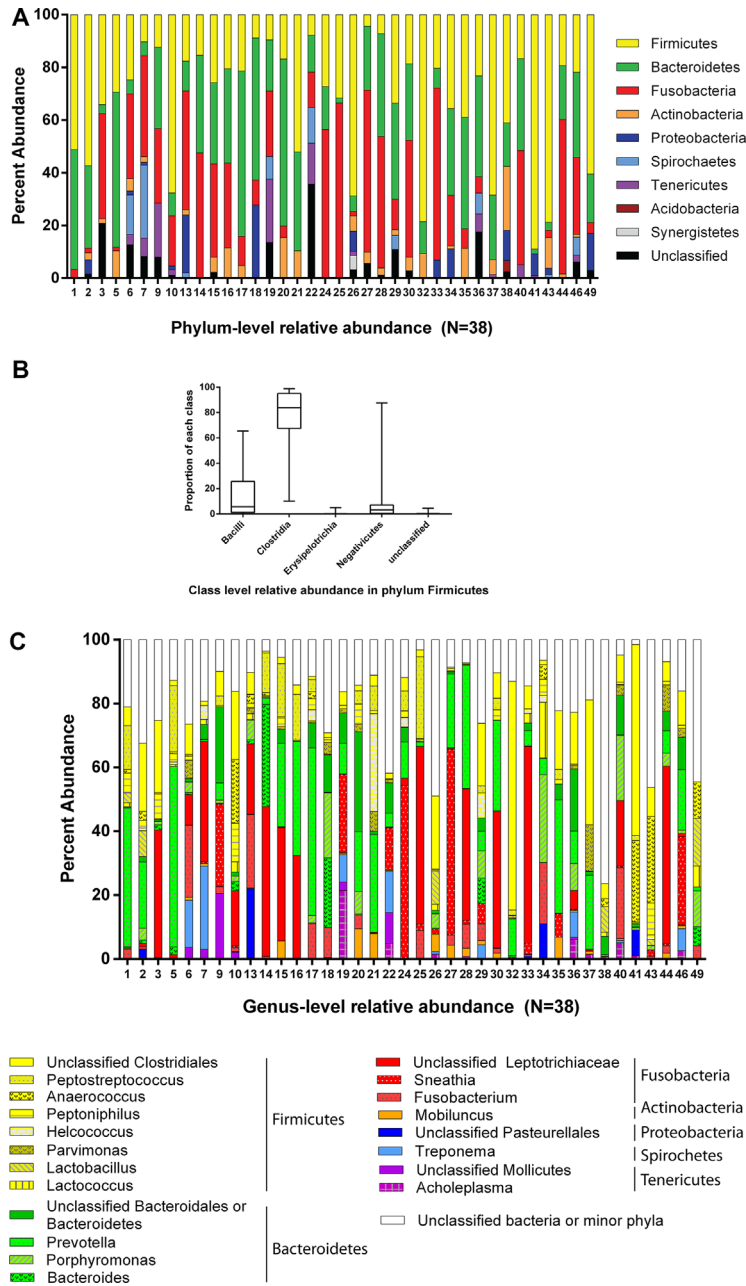
Fig. 1. A,B: Phylum-level relative abundance within vaginal microbial communities of all 38 baboons (A) and average relative abundance for the Phylum Firmicutes at class level (B). A: The phyla ≥1% are individually represented. Individual animal numbers are indicated on the x-axis. "Unclassified" refers to OTUs not identifiable at the phylum level by comparison of available sequence to available phylogenetic databases. B: Clostridia is the predominant class in Firmicutes in baboons instead of Bacilli, which includes Lactobacillus. Whiskers indicate the range. Within the box, the central horizontal line indicates median, and top and bottom lines indicate 75th percentile and 25th percentile of relative abundance, respectively. C: Genus-level relative abundance within vaginal microbial communities of all 38 baboons. Only the genera ≥1% are individually represented in this graph. Genera are color coded by phylum, and are represented by yellow (Firmicutes), green (Bacteroidetes), red (Fusobacteria), orange (Actinobacteria), dark blue (Proteobacteria), light blue (Spirochetes) and purple (Tenericutes). White includes unclassified bacteria and minor phyla (all the genera present at <1% were grouped here). Individual animal numbers are indicated on the x-axis. "Unclassified" refers to OTUs not identifiable below the indicated level by comparison of available sequence to existing phylogenetic databases. "Unclassified" bacteria at the phylum level were grouped with "minor phyla" (phyla present <1%).

TABLE II. Twenty Most Prevalent Genera in Vaginal Microbial Communities of 38 Wild-Caught, Captive Baboons

Genus	% total sequences[a]	% animals with this genus[b]
Prevotella	14.05	71
Sneathia	6.06	34
Fusobacterium	4.06	45
Peptostreptococcus	3.73	42
Porphyromonas	3.56	42
Anaerococcus	2.71	26
Treponema	2.24	18
Bacteroides	2.10	24
Peptoniphilus	2.01	45
Helcococcus	1.50	21
Parvimonas	1.41	32
Mobiluncus	1.39	26
Lactobacillus	1.25	16
Acholeplasma	1.18	21
Lactococcus	1.07	24
Cronobacter	0.81	5
Aerococcus	0.75	13
Facklamia	0.74	11
Streptococcus	0.70	11
Unclassified	38.21	100

[a]Average fraction of sequences across 38 baboons.
[b]Fraction of baboons with $\geq 1\%$ of total sequences.

Therefore, we only considered differences that were detectable by all three methods as significant.

Community Analysis Within Subgroups: Richness, Evenness, and Shannon Diversity Index

Community parameters of diversity, richness, and evenness within each subgroup were evaluated (Table IV). The Shannon diversity index, was significantly lower in the adults than in subadults ($P = 0.035$). Microbial community diversity is dependent on both the richness (number of OTUs) and evenness (relative abundance of OTUs) within a microbial community. The decreased diversity seen in the adult baboons was due to decreased community evenness rather than changes in richness, as reflected by significantly lower Shannon evenness scores in the adults ($P = 0.028$) but no difference in observed OTUs (S_{obs}) or predicted richness (Chao1 richness estimator). In the reproductive cycle and menstruation subgroups, there were no significant differences in richness (OTUs and Chao 1 richness estimator), evenness (Shannon evenness scores) or diversity (Shannon diversity index) (Table IV).

Community Analysis Within Subgroups: Relative Abundance of Specific Phyla

The vaginal microbial communities of each subgroup were evaluated with respect to relative

abundance at the phylum level. The same three phyla (Firmicutes, Bacteroidetes, and Fusobacteria) predominated in each subgroup (Fig. 3). The phyla present in lower abundances included Tenericutes, Proteobacteria, Spirochetes, and "unclassified" phyla, which represented OTUs that could not be matched at the phylum level. For the age subgroup, the lower abundance phyla, when considered together, were present in significantly higher abundance in subadult than adult animals ($P = 0.027$) (Fig. 3A). Additionally, the major phylum Bacteroidetes was present in lower abundance in subadult than in adult animals ($P = 0.045$), consistent with overall greater evenness across phyla in the subadults. In the menstruation or reproductively cycling subgroups, there were no differences in relative abundance for any phyla (Fig. 3B and C).

Metagenomic Evaluation: Comparison by Predicted Functional Content

To this point, we had evaluated the vaginal microbial community by its structure (membership and relative abundance) and by measures of diversity. The overall picture was of highly diverse microbial communities with high inter-individual variation between baboons, in marked contrast to the low diversity, relatively uniform, *Lactobacillus*-dominant vaginal microbial community of humans. One possible explanation for the apparently high inter-individual variation in baboons is that a variety of microbial communities could perform the same functional roles in this environmental niche. If this is the case, comparison across baboons based on functional analysis might reveal greater uniformity than is evident by community analysis.

Analysis of predicted functional content of microbial communities was performed using PICRUSt and HUMAaN to calculate the relative abundances of predicted functional metabolic pathways (modules) [Abubucker et al., 2012; Langille et al., 2013; Muto et al., 2013]. Surprisingly, despite the inter-individual variation and high diversity that had been evident in comparing community structures, the relative abundance of the majority of modules was strikingly similar across the animals (Fig. 4A). Additionally, comparison of the adults ($N = 14$) and subadults ($N = 9$) showed no significant difference in the average relative abundance of any of the modules (unpaired t-test, $P > 0.05$, data not shown). The individual modules are identified and their relative abundances (mean $\pm$ SD) across all baboons are shown in Figure 4B. Although these data must be interpreted with caution, as they reflect predicted rather than measured function, these findings suggest that varying microbial community compositions can potentially serve the same functional role.
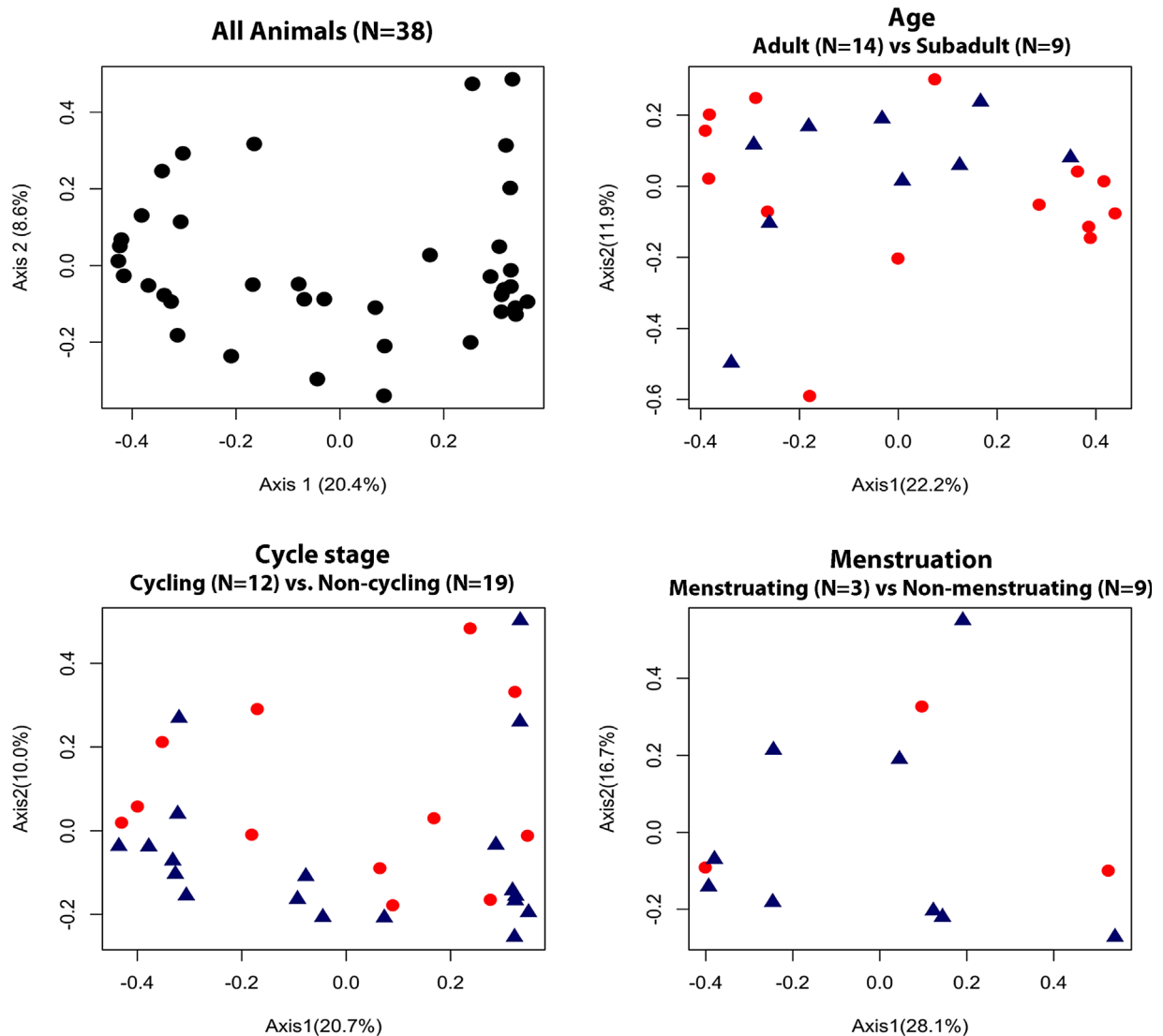
Fig. 2. A–D: PCoA of θ_{YC} distances between baboon vaginal bacterial communities. Variance explained by the first two axes (Axis 1 and 2) are shown in the figures. The number of axes generated were 36 (all animals), 21 (age comparison), 29 (cycle stage comparison) and 10 (menstruation comparison). A: All 38 animals. Although there is some visual separation of data on axis 1 and 2, these were not statistically confirmed as true clusters using Dirichlet Multinomial Mixtures (see text). B: Age comparison including adults ($N = 14$, red circle) and subadults ($N = 9$, blue triangle). C: Cycle stage comparison including cycling ($N = 12$, red circle) and non-cycling ($N = 19$, blue triangle) animals. D: Menstruation comparison including menstruating ($N = 3$, red circle) and non-menstruating ($N = 9$, blue triangle) animals. There were no significant differences between groups by AMOVA performed on θ_{YC} distances ($P < 0.05$, see Tables).

DISCUSSION

The goal of this study was to evaluate the influence of age, reproductive cycle stage, and menstruation on the vaginal microbiome of wild-caught, captive baboons. The differences in microbial compositions between baboons and humans are significant despite the similarities between these species in anatomical structures and reproductive physiology. The broad significance of evaluating the effects of age and reproductive cycle in the baboon vaginal microbiome, with comparison to the human vaginal microbome, is the potential to understand differing strategies by which host–microbial ecosystems adapt to these physiologic stages.

Our results corroborated earlier findings that the vaginal microbiome of the baboon is highly diverse as compared to humans [Hashway et al., 2014; Rivera et al., 2010, 2; Stumpf et al., 2013]. Although cross-study comparisons must be viewed with caution, the average number of OTUs (at 3% difference) per baboon in this study was 42.1. The average number of OTUs (at 3% difference) previously reported in asymptomatic women was 25 [Oakley et al., 2008]. Interestingly, the average OTUs for baboons in our study was lower than that previously reported in
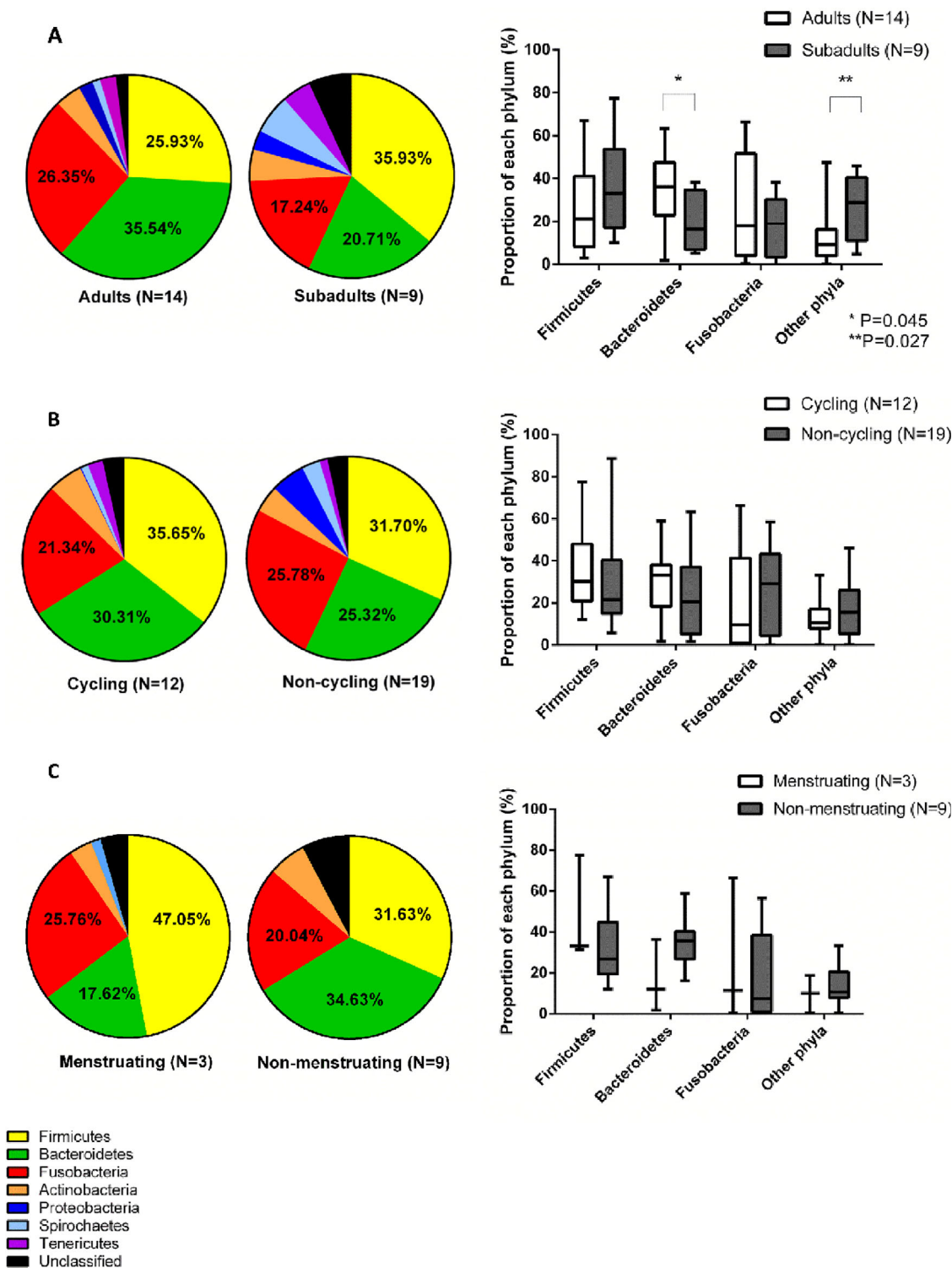
Fig. 3. Comparison of vaginal microbial communities based on age (A), cycle stage (B) and menstruation (C). Average relative abundance at the phylum level is represented in pie charts. Intraphylum variations are represented in box and whisker plots. Whiskers indicate the range. Within the box, the central horizontal line indicates median, and top and bottom lines indicate 75 percentile and 25 percentile of relative abundance, respectively. Other phyla are synonymous with the "non-dominant" phyla described in the text and include all phyla except Firmicutes, Bacteroidetes and usobacteria. Significant difference were found in the abundance of phylum Bacteroidetes (*) and other phyla (**) between adults and subadults (Fig. 3A, age comparison) with P-values of 0.045 and 0.027, respectively. Only significant P-values are reported.

TABLE III. Vaginal Microbial Community Structure Comparisons Within Subgroups

Subgroup comparison	AMOVA[a]	UniFrac$_{unweighted}$[b]	UniFrac$_{weighted}$[c]
Age (adult vs. subadult)	$P = 0.25$	$P = 0.429$	$P < 0.05$
Cycle (menstruating vs. non-menstruating)	$P = 0.84$	$P = 0.487$	$P < 0.05$
Cycle (cycling vs. non-cycling)	$P = 0.48$	$P = 0.959$	$P < 0.05$

[a]θ_{YC} dissimilarity index, OTU-based comparison.
[b]unweighted UniFrac, phylogeny-based comparison.
[c]weighted UniFrac, phylogeny-based comparison.

women with bacterial vaginosis (61) [Oakley et al., 2008]. However, the wide inter-individual range of OTUs per baboons (18.7–196.0) and the large number of total identified OTUs (781) showed that there is great variation in the "normal" microbial community in this species.

Recently, attempts have been made at applying theories of microbial ecology towards the interpretation of differences in host-microbial ecosystems [Costello et al., 2012; Hickey et al., 2012; Robinson et al., 2010; Stumpf et al., 2013; Yeoman et al., 2011]. In terms of vaginal microbial diversity, humans appear distinct among primates. A recent survey of the vaginal microbiome across nine primate species (including human) showed that only humans had a marked preponderance of *Lactobacillus* at this site [Yildirim et al., 2014]. One explanation for this difference is that NHPs may rely on a different predominant mechanism of protection from pathogens in the vagina. Specifically, rather than antimicrobial effects of lactic-acid derived low pH, baboons and other NHPs may rely on high diversity as a means of competitive niche exclusion. In environmental microbial ecology, high community diversity is classically associated with greater overall ecosystem stability [Hickey et al., 2013; McCann, 2000; Robinson et al., 2010]. Two principles that contribute to this effect are functional redundancy and complementary resource use. Functional redundancy refers to functional overlap between some community members so that ecosystem stability is maintained in case of species loss [McCann, 2000; Rosenfeld, 2002]. Complementary resource use, on the other hand, refers to the occupation of separate functional or microenvironmental "niches" by different mem-

bers within the ecosystem, thus decreasing resource competition by any one member [McCann, 2000; Yachi & Loreau, 2007]. By a combination of these strategies, high diversity communities may competitively exclude invasive outside species.

If high community diversity and niche exclusion were the major protective strategies used for pathogen protection in the vaginal microbiome, it might be expected that puberty and the onset of sexual activity would be associated with an increase in microbial diversity. Instead, we found a decrease in diversity between the vaginal communities of adults and subadult animals, corresponding to a decrease in species evenness. A similar directional change, although of greater magnitude, is seen in humans. While the vaginal microbiome of young, pre-pubertal girls is highly diverse, the peri-pubertal decline in diversity eventually results in the *Lactobacillus*-dominant community state seen in most adult women [Hammerschlag et al., 1978; Hickey et al., 2012; Linhares et al., 2011; Yamamoto et al., 2009]. This would seem to contradict the theories above and suggest that lower diversity is advantageous in the post-pubertal environment. One potential explanation is that maintaining a high diversity community may be an energy-expensive strategy, particularly in a low-resource environment such as the relatively nutrient-poor vaginal ecosystem [Stumpf et al., 2013]. The energy costs can be tempered by the principle of competitive inhibition, which indicates that fewer species are necessary to maintain ecosystem stability if the species present represent a larger variance in function [McCann, 2000; Yachi & Loreau, 2007]. Thus, selection occurring at puberty may represent pruning of potential community members

TABLE IV. Vaginal Microbial Community Parameter Comparisons[a] Within Subgroups

	Shannon diversity index[b]	OTU	Chao1 (richness)	Shannon evenness
Adult ($N = 14$)	2.05 ± 0.46[*]	38.7 ± 17.6	55.4 ± 32.9	0.57 ± 0.11[**]
Subadult ($N = 9$)	2.54 ± 0.58[*]	42.1 ± 13.5	54.0 ± 20.2	0.69 ± 0.13[**]
Menstruating ($N = 3$)	1.99 ± 0.95	36.8 ± 7.84	54.0 ± 23.8	0.55 ± 0.24
Non-menstruating ($N = 9$)	2.02 ± 0.43	33.2 ± 11.8	45.0 ± 17.4	0.58 ± 0.09
Cycling ($N = 12$)	2.02 ± 0.55	34.1 ± 10.7	47.2 ± 18.4	0.57 ± 0.13
Non-cycling ($N = 19$)	2.24 ± 0.54	37.9 ± 11.6	47.4 ± 14.1	0.63 ± 0.13

[a]Data reported as mean $\pm$ SD.
[b]Shannon diversity index is logarithmically proportional to true diversity.
[*]$P = 0.035$.
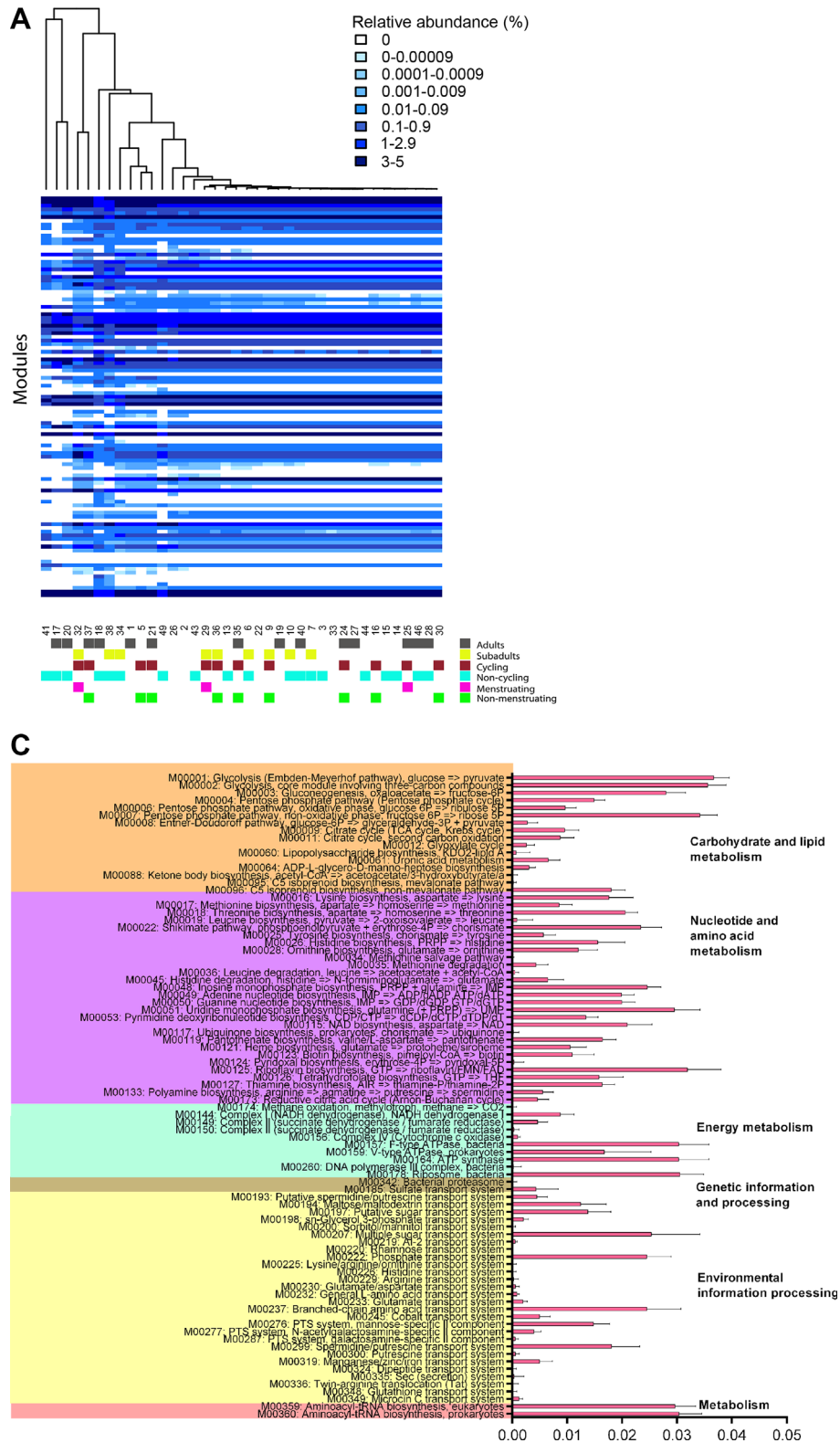[**]$P = 0.028$ (P-values only reported for statistically significant comparisons).

Fig. 4. A: Predicted metagenomic functions for all 38 baboon samples. The heat map shows the relative abundance of predicted gene pathways for all animals ($N = 38$). Columns represent individual animals and rows represent individual gene pathways (Modules). The color bars below indicate subgroups (adult vs. subadult, cycling vs. non-cycling, and menstruating vs. non-menstruating) in which individual animals were included. B: Predicted metagenomic functions for all 38 baboon samples. Relative abundance of functional metabolic pathways (shown as KEGG modules) was calculated using HUMAaN and grouped into major functional categories. Bar graphs represent average relative abundance of pathways (Mean $\pm$ SD) at $\geq 10^{-4}$.

down to the number necessary to occupy available environmental niches but minimize cost of excessive functional redundancy. One disadvantage of our study was that only peripubertal animals were available for evaluation. Evaluation at an earlier time point (pre-pubertal animals) may have detected larger effects and provided a better assessment of puberty as a critical time point in the formation of the vaginal microbial community.

Interestingly, there were no significant differences in vaginal microbial community structures correlated with menstruation or cycle stage using two of three measures (Yue Clayton dissimilarity index and unweighted UniFrac vs weighted UniFrac). In women, highest community constancy has been associated with estrogen-dominated periods (follicular phase) of the reproductive cycle [Gajer et al., 2012]. In addition, menses has been associated with decreased community stability, yet not with altered diversity [Gajer et al., 2012; Hickey et al., 2013; Lopes dos Santos Santiago et al., 2011]. In our study, interpretation of menstruation and cycling data was challenging for several reasons. First, sampling was limited to the distribution of reproductive cycle stages present in the animals at a single time point. Thus, only three animals were in menses at the time of sampling, resulting in a small group size that likely made it difficult to detect any but the most extreme differences. Similarly, small numbers of animals in each stage of the menstrual cycle necessitated grouping into cycling and non-cycling animals. More precise animal grouping within the reproductive cycle may have enabled detection of more nuanced differences with reproductive cycle phase. With these caveats in mind, our results suggest that reproductive cycle hormonal fluctuations are not major influences on the composition of vaginal microbial communities in the baboon.

With respect to specific composition at the genus level, the vaginal microbiome of the seemingly healthy baboons in this study contained many taxa associated with the dysbiotic state of "bacterial vaginosis" (BV) in women, a condition correlated with vaginal discharge, higher susceptibility to sexually transmitted infections, and preterm birth [Gajer et al., 2012; Marrazzo et al., 2010; Ravel et al., 2011; Romero et al., 2004]. For example, the genera *Prevotella* (most abundant genus in baboons for this study), *Sneathia*, and *Mobiluncus* are typically considered pathogenic in women, yet were not associated with discharge or other signs of disease in baboons in this study. However, the presence of pathogenic species does not necessarily correlate with disease. For example, many apparently healthy, asymptomatic women with highly diverse vaginal microbial communities would meet clinical criteria of BV due to the presence of increased strict anaerobes, higher vaginal pH,

and decreased numbers of lactobacilli [Oakley et al., 2008; Ravel et al., 2011, 2013; Yildirim et al., 2014]. Furthermore, our sequence data did not permit evaluation to the species or subspecies level. For instance, numerous *Fusobacterium* were present. This is a genus that contains pathogenic species and has been detected in women with BV [Twin et al., 2013]. However, *Fusobacterium nucleatum* is a commensal in the oral cavity, and has been associated with induction of antimicrobial and immunomodulatory peptides in oral epithelium [Yin & Dale, 2007]. It is thus possible that, at the species level, members of the diverse taxa of the baboon vaginal microbiome are actually contributing to ecosystem stability or protective interactions with the immune system. It is also possible that some baboons in our study, despite the absence of clinical signs, are in a disease susceptible state. For example, *Treponema* was found in 6/9 (66.7%) subadult animals and 7/14 (50%) adult animals. Although we were unable to speciate *Treponema* in this study, *Treponema pallidum* has been found as a naturally occurring pathogen associated with necroulcerative genital lesions in wild baboons in Tanzania [Harper et al., 2012]. Future evaluation using full length 16S rRNA sequencing and bacterial culture may contribute to species identification and determining whether these species play a pathogenic or commensal role.

Despite the high inter-individual differences among baboons, the microbial communities looked similar across all animals when functionality was investigated using a predictive method (PICRUSt) [Langille et al., 2013]. This method has not been validated for baboon-origin microbial communities and it is possible that there are inaccuracies based on comparison to available databases. Additionally, these findings represent predicted functionality, not actual gene expression data. The results nevertheless raise the possibility that compositionally different microbial communities have similar functions, as previously demonstrated in the intestinal tract [Theriot et al., 2014]. Additionally, some women lacking vaginal lactobacilli have been shown to have similar levels of lactic acid production by other bacteria, such as *Streptococcus* spp. or *Atopobium* spp. [Farage et al., 2010; Gajer et al., 2012; Lamont et al., 2011]. The future use of gene expression data to complement community structure data may uncover functional similarities that are not evident when comparing community structure alone.

Our results indicate that, although the overall baboon vaginal microbiome is diverse, there is a significant decrease in diversity in adults versus subadult animals due to reduction in the percentage of the lower abundance taxa. This may represent peripubertal host selection of a community structure optimized for post-pubertal host health. Additionally, analysis of predicted functional pathways

suggests that there is some functional equivalence across the seemingly disparate communities seen in individual baboons. Future studies in baboons or other NHPs focusing on microbial species-level comparisons and evaluation of gene expression patterns may contribute to greater understanding of the mechanisms employed by a high diversity vaginal microbiome in supporting adult reproductive functions and host health.

ACKNOWLEDGMENTS

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