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RESEARCH ARTICLE

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The microbial reproductive ecology of white-faced capuchins (*Cebus capucinus*)

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Changes in reproductive status influence energy and nutrient requirements in female primates. The gut microbiota may buffer changes in energy demands, with shifts in community composition increasing the energy production potential of the gut during pregnancy and lactation. In this study, we examine changes in the gut microbiome of wild, female white-faced capuchins (*Cebus capucinus*) across different reproductive states. Fecal samples (n = 39) were collected from five adult females over the course of a year. Gut microbial community composition was assessed using 16S rRNA gene sequences, and PICRUSt was used to make metagenomic functional predictions. We found a significant relationship between reproductive state and both the structure and predicted function of the gut microbiome, neither of which were associated with host diet. For example, the relative abundance of Firmicutes was significantly lower in lactating females compared with cycling females; the relative abundance of Actinobacteria was significantly higher in pregnant females compared with lactating females, and there was a trend toward higher relative abundances of Proteobacteria in pregnant females compared with cycling females. The results of this study suggest that, in addition to behavioral and dietary adaptions, the gut microbiota may play a role in allowing female primates to meet their changing energetic needs during reproduction. Further studies of the "microbial reproductive ecology" of primates will help advance our understanding of gut microbial contributions to primate energetics.

KEYWORDS
energetics, gut microbiome, reproduction, white-faced capuchins

1 | INTRODUCTION

Pregnancy and lactation are periods of energetic stress for female primates. In mammals, pregnancy increases daily energy expenditure by 20–30%, and lactation increases daily energy expenditure by 37–39% (Aiello & Wells, 2002). In humans, women increase their energy intake during pregnancy by 8–10%, and lactating women increase their energy intake by 26% (Dufour & Sauther, 2002). Captive *Papio anubis* increase their energy intake by 11–27% during lactation (Dufour & Sauther, 2002). Similarly, protein requirements during lactation in both human and nonhuman primates increase by at least one-third, with adult non-reproductive primates requiring 5–8% of their metabolizable energy to be protein, and pregnant or lactating females requiring 10% of their metabolizable energy to be protein (Oftedal, 1991).

Across evolutionary timescales, primates have developed behavioral and physiological strategies that allow them to adjust to these increased energy and nutrient requirements during pregnancy and lactation. These strategies include incorporating more high-protein and high-fat foods (e.g., animal prey, underground storage organs) into their diets, decreasing daily fetal growth costs by extending gestation, and/or increasing metabolic efficiency (Aiello & Wells, 2002; Dufour & Sauther, 2002). Additionally, over a single lifespan, individual primates

may increase energy intake during periods of pregnancy and lactation by accelerating the rate of food intake, switching to higher-quality foods, or increasing time spent feeding. For example, *Aotus azarai* and *Saguinus fuscicollis* both increase consumption of insect prey while lactating (Rothman, Raubenheimer, Bryer, Takahashi, & Gilbert, 2014). Pregnant and lactating primates are also reported to utilize body tissue stores, decrease basal metabolic rate, reduce energy expenditure, and time peak demands to coincide with seasonal peaks in food availability (Dufour & Sauther, 2002; Lee, 1987; Rothman et al., 2014).

Recent research implicating the gut microbiome in host nutrition reveals another potentially important mechanism by which female primates may compensate for the increased nutritional demands of reproduction. The gut microbiota processes otherwise indigestible food resources, such as plant structural carbohydrates, and increases nutrient uptake in the gut (Cummings & Macfarlane, 1997; Hooper, Midtvedt, & Gordon, 2002). Therefore, differences in the composition and function of the gut microbiome have been associated with differences in host digestive efficiency and nutritional status in a range of contexts (Claesson et al., 2012; De Filippo et al., 2010; Hildebrandt et al., 2009; Nelson, Rogers, Carlini, & Brown, 2013; Wu et al., 2011). For example, higher ratios of Firmicutes to Bacteroidetes have been associated with increased breakdown of plant structural carbohydrates and production of short chain fatty acids, which can readily be used as an energy source by the host (Turnbaugh et al., 2009). These shifts in the gut microbiota may be triggered by factors such as changes in host diet or hormone levels, and, if timed correctly, could help hosts compensate for energy shortfalls and changes in energy expenditure (Amato, 2016). Indeed, a study of *Alouatta pigra* found that reproductive state significantly altered gut microbial community structure (Amato & Righini, 2015; Nakamura et al., 2011; but see Ren, Grieneisen, Alberts, Archie, & Wu, 2016).

Despite the potential nutritional advantages altered gut microbiota composition and function might provide to hosts during pregnancy and reproduction, there are also likely to be trade-offs. In humans, changes in the gut microbiome that increase metabolic efficiency also appear to increase inflammation (Koren et al., 2012). Furthermore, given that several strains of *Bacteroides* (Bacteroidetes) are beneficial to the host immune system (Keeney & Finlay, 2011; Round & Mazmanian, 2009), increased ratios of Firmicutes to Bacteroidetes are likely to positively affect host nutrition but negatively affect host immune response. Thus, altering the gut microbiome in this way may be beneficial for individuals who experience short term energetic shortfalls, such as pregnant and lactating females, but it may not be a sustainable long term strategy for primates.

Capuchin monkeys (*Cebus* spp. and *Sapajus* spp.) provide an excellent model for investigating the potential role of the gut microbiota in helping female primates meet the nutritional demands of pregnancy and lactation. In capuchin monkeys, both infant and maternal energetic needs are particularly high during lactation. Capuchins not only have small brains for their body size at birth relative to other primates (Elias, 1977; Hartwig, 1996; Phillips & Sherwood, 2008), but they also have unusually fast post-natal brain growth for a non-ape primate (Hartwig, Rosenberger, Norconk, & Owl, 2011; Isler et al., 2008; Phillips & Sherwood, 2008). Most studies of white-faced capuchins have occurred in seasonal dry forests or tropical rainforests, where rainfall (800–2600 mm annually) occurs primarily during a May–December wet season, and the abundance of fruit is correlated with rainfall (Crofoot, 2008; Fedigan & Jack, 2001). In these populations of *Cebus capucinus*, reproduction is highly seasonal, the period of peak fruit abundance coincides with mid-to-late lactation, and females modify their behavior and diet to compensate for changes in energetic requirements during pregnancy and lactation (Carnegie, Fedigan, & Melin, 2011). Specifically, females spend more time resting and less time foraging during the month when they give birth, and pregnant and lactating females spend more time consuming large invertebrates than cycling females (Rose, 1994). Additionally, lactating females have higher feeding rates and increased energy, protein, sugar, and fat intakes relative to cycling or pregnant females (McCabe & Fedigan, 2007).

The current study examines patterns in diet and gut microbiome composition and predicted function in female *C. capucinus* inhabiting an aseasonal tropical wet forest, in which temporal variation in resource availability, diet, and reproduction is limited relative to other sites. We examine whether: (i) the gut microbial community structure covaries with reproductive state; (ii) the predicted metagenomic function of the gut microbiome differs between each reproductive state; and (iii) the observed differences in gut microbial community structure can be attributed to changes in the energy harvest potential of the gut, measured as an increase in the ratio of Firmicutes to Bacteroidetes during pregnancy and lactation.

2 | METHODS

2.1 | Field methods

The study took place at La Suerte Biological Field Station (LSBFS) in northeastern Costa Rica (10.445N, 83.784W) from January 2013 through January 2014. LSBFS is classified as an aseasonal tropical wet forest. During the study period, annual rainfall was 3,116 mm, average monthly rainfall was 240 ± 145 mm (range = 67–541 mm), and average monthly temperature was 29.8 ± 1.6 C (range 26.6–31.9 C). There is limited seasonal variation in the percentage of total feeding and foraging time spent consuming fruit and invertebrates at this site. In a given month during the study, fruit consumption ranged from 30.9% to 60.2% of total feeding and foraging time, and monthly invertebrate consumption ranged from 37.6% to 67.3% of total feeding and foraging time (Table 1). The percentage of time spend feeding and foraging on fruit was highest in May, October, and November, and lowest in January, February, and June (Table 1). Conversely, the highest percentages of time spent feeding and foraging on invertebrates occured in January, February, and June, and the lowest were in May, October, and November (Table 1). Rainfall is not correlated with fruit availability or invertebrate availability at this site (Mallott, 2016). While fruit and invertebrate consumption do not vary seasonally, the specific species of fruit and families of invertebrates consumed do vary throughout the year.

TABLE 1 Percentage of monthly feeding and foraging time spent on each type of food resources by white-faced capuchins at La Suerte Biological Field Station from February 2013 to January 2014

	Fruit (%)	Flowers (%)	Seeds (%)	Leaves (%)	Invertebrates (%)	Vertebrates (%)	Other (%)
February	42.0	0.9	0.0	0.3	55.2	0.0	1.6
March	51.0	0.0	0.0	0.4	48.2	0.0	0.4
April	47.2	0.5	0.0	1.1	50.9	0.0	0.3
May	53.5	0.0	0.2	0.5	45.8	0.0	0.0
June	30.9	1.5	0.0	0.0	67.3	0.0	0.3
July	46.8	3.0	0.0	0.6	49.1	0.0	0.5
August	48.0	3.3	0.0	0.0	47.8	0.0	0.9
September	43.4	0.7	0.0	0.0	54.7	0.2	1.0
October	60.2	1.3	0.0	0.5	37.6	0.0	0.4
November	53.0	2.2	0.0	0.7	43.6	0.0	0.5
December	47.3	1.1	0.0	1.5	49.8	0.0	0.3
January	41.5	0.9	0.0	0.0	57.6	0.0	0.0
Overall	47.8	1.2	0.01	0.5	49.8	0.01	0.7

We observed five adult females from one group of 21–22 habituated, individually recognizable *C. capucinus*. The group contained four adult males, five adult females, eight to nine juvenile males, two to three juvenile females, and zero to two infants during the study period. During 1,341 hr of observation, 575 hr of dietary data (observed feeding and foraging bouts) were collected using 1 hr focal follows. Dietary items (ripe fruit, unripe fruit, invertebrates, vertebrates, flowers, leaves, seeds, other) were recorded every 2 min during foraging bouts, and when possible, consumed food items were identified to the level of species (for fruit) or Order (for invertebrates). The percentage of total feeding and foraging time exploiting fruits or invertebrates was calculated for each female across a two-week period. Dietary composition for fruit at the species level was calculated over a two-week period as the minutes per hour an individual spent consuming a specific species of fruit. Invertebrate dietary composition was calculated using the relative abundance of DNA sequences assigned to a family of invertebrates within each individual fecal sample (Mallott, Garber, & Malhi, 2017). Additional details on the foraging behavior and dietary data collection for this group during the time period of the study have been published previously (Mallott, 2016; Mallott, Amato, Garber, & Malhi, 2018; Mallott et al., 2017).

During the study period, two females were cycling, then pregnant, and then lactating; a third female was lactating and then cycling; a fourth female was cycling and then pregnant; and a fifth female was cycling for the duration of the study (Figure 1). As four of the five adult

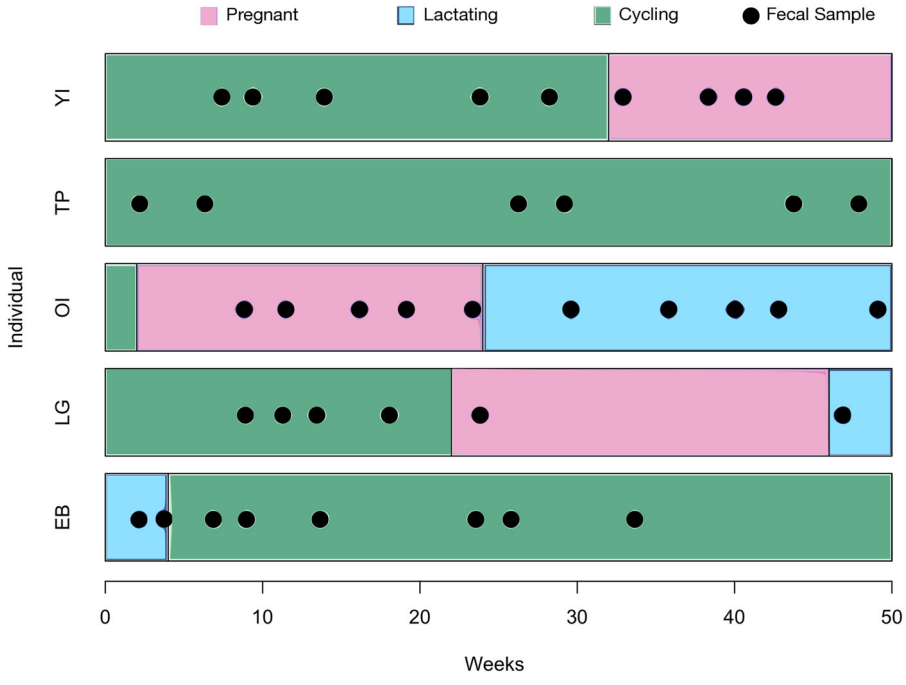

FIGURE 1 Reproductive status over the course of the study period and timing of fecal sampling for each individual

females fell into more than one reproductive status category during the study period, we were able to compare changes between reproductive states within the same individual. Females were classified into reproductive states based on observational data. The end of lactation was defined as the lack of observed nursing behavior. Pregnancy was defined retrospectively as ~5.5 months prior to birth based on reported gestation length in white-faced capuchins (average 157.83 ± 8.13 days, range 145–166 days) (Carnegie, 2011). Cycling females were defined as those females not pregnant or lactating. We did not have the hormonal data to confirm reproductive states, and may not have captured pregnancies that did not result in a full-term offspring or non-reproductively active periods in our data. Additionally, due to the limitations of our small sample size, we are not able to distinguish between early, mid, and late lactation or the first, second, and third trimesters of pregnancy. Thus, we are not capturing changes in the gut microbiome in response to variation in energetic demands within pregnancy or lactation.

Fecal samples were collected from all five females throughout the study period. Out of 39 total fecal samples, 21 samples were collected from five females while they were cycling (four to six samples from each), ten samples were collected from the three females who were pregnant during part of the study period (one to five samples per individual), and eight samples were collected from the three females who were lactating for a portion of the study period (one to five samples per individual). Each female was sampled between 6 and 10 times over the course of the study (Figure 1). Fecal samples were collected directly following defecation in sterile 15 ml tubes; 90% ethanol was added to the tubes immediately after fecal samples were collected; and the samples were frozen at −20 C within 12 hr.

All data collection methods were approved by the University of Illinois IACUC, and La Suerte Biological Field Station, MINAET, SINAC, and CONAGEBIO in Costa Rica. Appropriate import permits were obtained from the CDC and USDA. The research adhered to the legal requirements of Costa Rica and the American Society of Primatologists' Principles for the Ethical Treatment of Primates.

2.2 | Molecular methods

DNA was extracted from all samples using a QIAamp DNA Stool Mini Kit following established protocols (Mallott et al., 2017). This extraction method relies on chemical lysis instead of physical disruption, and the results may not be directly comparable to gut microbiome studies that use bead-beating-based DNA extraction methods. After DNA extraction, a two-step polymerase chain reaction (PCR) amplification was performed. In the first PCR, the V4 region of the 16S rRNA gene was amplified using the 515f and 926r Earth Microbiome Project primers (www.earthmicrobiome.org) that had a Fluidigm CS1 or CS2 linker sequence added to the primer sequence. The first PCR reaction was carried out in a total volume of 25 µl, consisting of 2 µl of DNA sample taken directly from the DNA extraction step, 12.5 µl of 2X Phusion HF Mastermix, 1.25 µl of 10 µM forward primer, 1.25 µl of 10 µM reverse primer, and 8 µl of molecular grade H_2O. The following PCR program was used: 3 min at 98 C; 28 cycles of 30 s at 98 C, 45 s at 55 C, and 45 s at 72 C; 1 min at 72 C, and hold at 4 C indefinitely. Successful amplification of the PCR products was verified using a 1% agarose gel, with all samples producing strong bands. Each sample was amplified in triplicate and then combined prior to the second PCR amplification. A second amplification was performed using the Fluidigm AccessArray primers containing the CS1 or CS2 linker sequences, sample-specific barcode sequences, and Illumina sequencing adapters. The second PCR reaction was carried out in a total volume of 20 µl, containing 1 µl of DNA added directly from the combined product from the first PCR reaction, 10 µl of 2X Phusion HF Mastermix, 4 µl of 0.4 µM Fluidigm AccessArray Barcoded primers for Illumina, and 5 µl of molecular grade H_2O. The following PCR program was used: 5 min at 98 C; 8 cycles of 30 s at 98 C, 30 s at 60 C, 45 s at 72 C; hold at 4 C indefinitely. Extraction and PCR negatives were used to control for contamination. PCR products were purified and normalized using a SequalPrep Normalization Plate, and sequenced on the Illumina MiSeq V3 platform at the University of Illinois Chicago DNA Services Facility. Raw DNA sequences are available in the Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under BioProject ID PRJNA423292.

2.3 | Sequence processing and analysis

Sequencing yielded 638,145 sequence reads (average of 16,787.39 per sample, range of 8,427–45,909). One sample that yielded only 54 sequences was excluded from further analysis. Paired sequences were merged with PEAR 0.9.6 (Zhang, Kobert, Flouri, & Stamatakis, 2014), and sequences were trimmed and quality-filtered in QIIME (Caporaso et al., 2010). Operational taxonomic units (OTUs) were picked open reference using the *sortmerna_sumaclust* algorithm and a 97% identity threshold. Taxonomy was assigned using the Greengenes 13_8 database in QIIME. OTU picking was performed on a larger dataset of 1,096 primate gut samples to improve OTU clustering and taxonomic assignments. All OTUs that were assigned to chloroplast genes were removed prior to downstream analysis. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict metagenomic function from the 16S rRNA sequencing data (Langille et al., 2013). The average weighted Nearest Sequence Taxon Index (NSTI) score, a measure of how closely related the bacteria in our sample are to known microbial genome sequences, was calculated for each sample to assess the quality of PICRUSt predictions. The NSTI for all samples was 0.089 ± 0.059 (range: 0.020–0.240), indicating that on average, there is a 9% difference between the sequence of a bacteria in our samples and the sequence of the bacteria that is the closest match in the reference database.

2.4 | Statistical analysis

Beta diversity calculations were carried out in R (Bray-Curtis dissimilarities) and in QIIME (unweighted and weighted UniFrac distances). Unweighted and weighted UniFrac distances were calculated in QIIME using the *core_diversity_analyses.py* script,

rarefying the samples to an even sampling depth of 5,982 sequence reads per sample. Bray-Curtis dissimilarity matrices were created by first calculating the relative abundance of OTUs in the chloroplast-filtered OTU table using the *decostand* function in the *vegan* package in R (Oksanen et al., 2017), next removing OTUs from the larger primate sample set not present in these samples, and finally using the *vegdist* function in *vegan* to calculate Bray-Curtis distances. Permutational multivariate analysis of variance (PERMANOVA) was used to compare the effect of reproductive status on dietary composition, gut microbial community composition, and predicted metagenomic function using Bray-Curtis distance matrices and the *adonis* function in the *vegan* package in R. Individual identity was included as a "strata" or blocking factor in the model to test for the effect of reproductive state within the same individual and to control for repeated measures. PERMANOVAs were also used to compare the effect of the percentage of feeding and foraging time spent on fruit and invertebrates on gut microbial community composition, including reproductive state as a variable and individual identity as a blocking factor in the model. Weighted and unweighted UniFrac distance matrices produced results that were similar to those using the Bray-Curtis distance matrices. Pairwise comparisons for PERMANOVA results were computed using the *pairwiseAdonis* package in R (Martinez Arbizu, 2017), with modifications to account for the use of a blocking factor in the model. Nonmetric multidimensional scaling (NMDS) plots were used to visualize Bray-Curtis distances using the *vegan* package in R. Linear mixed-effects models were used to compare the relative abundance of phyla and the relative abundance of predicted metabolic function pathways using the *nlme* package in R (Pinheiro, Bates, DebRoy, Sarkar, & Team, 2017). Individual identity was included as a random effect in the linear mixed-effects models. Pairwise Tukey contrasts with Bonferroni correction for the linear mixed-effects models were carried out using the *multcomp* package in R (Hothorn, Bretz, & Westfall, 2008). Linear discriminant analysis effect size (LEfSe) was used to assess which taxa are over represented in one reproductive state compared to the other reproductive states, using individual as a sub-class to control for repeated measures (Segata et al., 2011). LEfSe uses Kruskal–Wallis sum-rank tests to identify differentially abundant taxa or pathways, then uses Wilcoxon rank-sum tests to perform pairwise comparisons between reproductive states, and estimates the effect sizes using linear discriminant analysis in order to determine which bacterial taxa or functional pathways are significantly over- or under-represented in a given reproductive state (Segata et al., 2011).

3 | RESULTS

The gut microbiota of white-faced capuchins contained 36 phyla and 570 genera. The dominant phyla were Proteobacteria (37.70%), Firmicutes (30.94%), Bacteroidetes (14.00%), and Actinobacteria (9.11%). Of the 4414 unique OTUs (434 ± 144 unique OTUs per sample) remaining after all filtering steps (see "section 2.4" above), 5.94% were not assigned to a known phylum.

As hypothesized, reproductive status of adult females was significantly related to gut microbial community structure ($F = 1.655$, $R^2 = 0.084$, $p = 0.023$), when controlling for individual identity (Figure 2). However, pairwise comparisons did not reveal significant differences between the gut microbiota of cycling and pregnant individuals ($F = 1.812$, $R^2 = 0.063$, $p = 0.040$, adjusted $p = 0.120$), cycling and lactating individuals ($F = 1.769$, $R^2 = 0.062$, $p = 0.045$, adjusted $p = 0.134$), or pregnant and lactating individuals ($F = 1.311$, $R^2 = 0.068$, $p = 0.178$, adjusted $p = 0.533$), but there is a trend toward cycling females differing from both pregnant and lactating females (Figure 2).

As diet could vary between reproductive states independent of dietary variation due to seasonality, we examined the effect of reproductive state on both foraging behavior and diet composition. In our study group, the percentage of feeding and foraging time that females spent on fruit or invertebrates, controlling for individual identity and reproductive state, was not significantly associated with gut microbial community composition ($F = 0.822$, $R^2 = 0.021$, $p = 0.560$

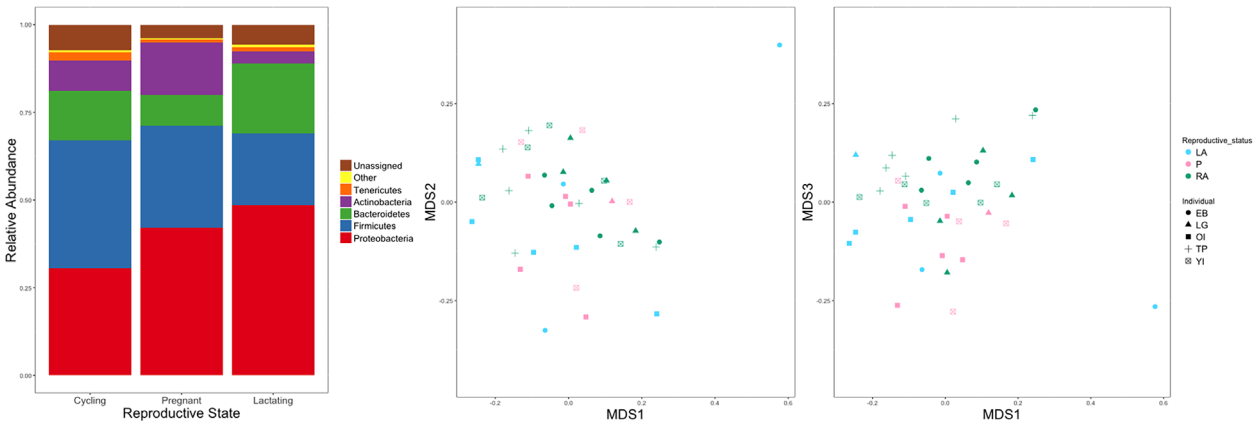

FIGURE 2 Relative abundance of bacterial phyla in the gut microbiota of *C. capucinus* across cycling ($n = 21$), pregnant ($n = 10$), and lactating ($n = 8$) states, and nonmetric multidimensional scaling (NMDS) plots using Bray–Curtis distances

and $F = 0.673$, $R^2 = 0.017$, $p = 0.751$). Additionally, species-level community composition of fruits consumed and family-level community composition of the invertebrates consumed were not significantly influenced by the reproductive status of individuals ($F = 1.432$, $R^2 = 0.035$, $p = 0.089$, and $F = 0.815$, $R^2 = 0.059$, $p = 0.901$). Thus, in our study group, diet was not strongly related to either gut microbial community composition or reproductive status, but reproductive status was related to gut microbial community composition.

In contrast to what we predicted, the ratio of Firmicutes to Bacteroidetes was not significantly different between reproductive states ($F = 0.505$, $p = 0.608$). However, the relative abundance of Firmicutes and Actinobacteria did differ significantly among reproductive states ($F = 6.312$, $p = 0.005$; $F = 4.133$, $p = 0.026$) and the effect of reproductive state on the relative abundance of Proteobacteria neared significance ($F = 3.023$, $p = 0.063$) (Figure 3). The relative abundance of Firmicutes was significantly lower in lactating individuals when compared with cycling individuals ($z = 3.515$, adjusted $p = 0.001$). The relative abundance of Firmicutes did not differ significantly between pregnant and cycling individuals or pregnant and lactating individuals ($z = 1.656$, adjusted $p = 0.221$; and $z = 1.688$, adjusted $p = 0.208$). There was a trend toward the relative abundance of Proteobacteria being higher in lactating individuals when compared with cycling individuals ($z = -2.276$, adjusted $p = 0.059$). The relative abundance of Proteobacteria did not differ significantly between pregnant and cycling individuals or pregnant and lactating individuals ($z = -1.634$, adjusted $p = 0.230$; and $z = -0.615$, adjusted $p = 0.811$). The relative abundance of Actinobacteria was significantly higher in pregnant individuals when compared with lactating individuals ($z = 2.849$, adjusted $p = 0.012$), but did not differ significantly between cycling and lactating individuals or between cycling and pregnant individuals ($z = 2.015$, adjusted $p = 0.108$; and $z = -1.263$, adjusted $p = 0.415$).

Linear discriminant analyses showed that each reproductive state was characterized by different bacterial taxa (Figure 4) Several taxa within Clostridia (Firmicutes) were significantly overrepresented in either cycling or pregnant reproductive states—Clostridiales (Firmicutes) was significantly overrepresented in cycling females and Lachnospiraceae (Firmicutes) and *Megasphaera* (Firmicutes) were significantly increased in pregnant females. Taxa within Actinobacteria were also significantly overrepresented in both cycling and pregnant females—*Collinsella* (Actinobacteria) was significantly overrepresented in cycling females and Microbacteriaceae (Actinobacteria) was significantly overrepresented in pregnant females. Pregnant females were also discriminated by increased relative abundances of *Stenotrophomonas* (Proteobacteria) and *Roseomonas* (Proteobacteria). Porphyromonadaceae (Bacteroidetes) was overrepresented in lactating females.

To confirm the patterns in the overall dataset, we examined shifts in the gut microbiome of each individual female across the study period. This analysis demonstrated individual differences in how the gut microbiome changes in response to female reproductive state (Figure 5). While we do not have enough data to test for statistical differences between reproductive states within a single individual, we do see that most females have increased relative abundances of Proteobacteria and decreased relative abundances of Firmicutes during more energetically expensive reproductive states, as well as increased relative abundances of Actinobacteria during pregnancy. This finding is not unexpected, as the overall pattern should reflect within-individual patterns given our small sample size. However, one individual, LG shows an atypical pattern, perhaps related to the death of her infant 1 week after birth.

Finally, as hypothesized, PERMANOVA showed that the predicted metagenomic function of the gut microbiome differed across reproductive states ($F = 2.171$, $R^2 = 0.110$, $p = 0.042$); however, pairwise comparisons did not reveal significant differences between any two reproductive states after adjusting for multiple comparisons. Linear mixed-effects models showed a significant influence of reproductive state on the relative abundance of pathways related to energy metabolism ($F = 4.708$, $p = 0.016$) and glycan metabolism ($F = 4.812$, $p = 0.015$). Pairwise comparisons indicated that the relative abundances of pathways related to energy metabolism were significantly lower in pregnant females compared with cycling females ($z = 3.029$, adjusted $p = 0.007$), and that the relative abundances of pathways related to glycan metabolism were significantly higher in

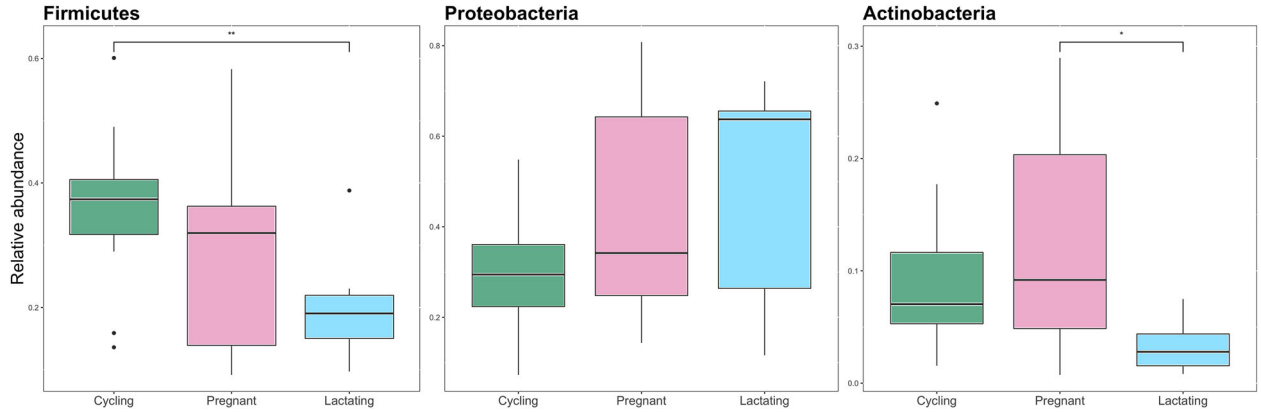

FIGURE 3 Relative abundance of Firmicutes, Proteobacteria, and Actinobacteria across cycling ($n = 21$), pregnant ($n = 10$), and lactating ($n = 8$) states in *C. capucinus*

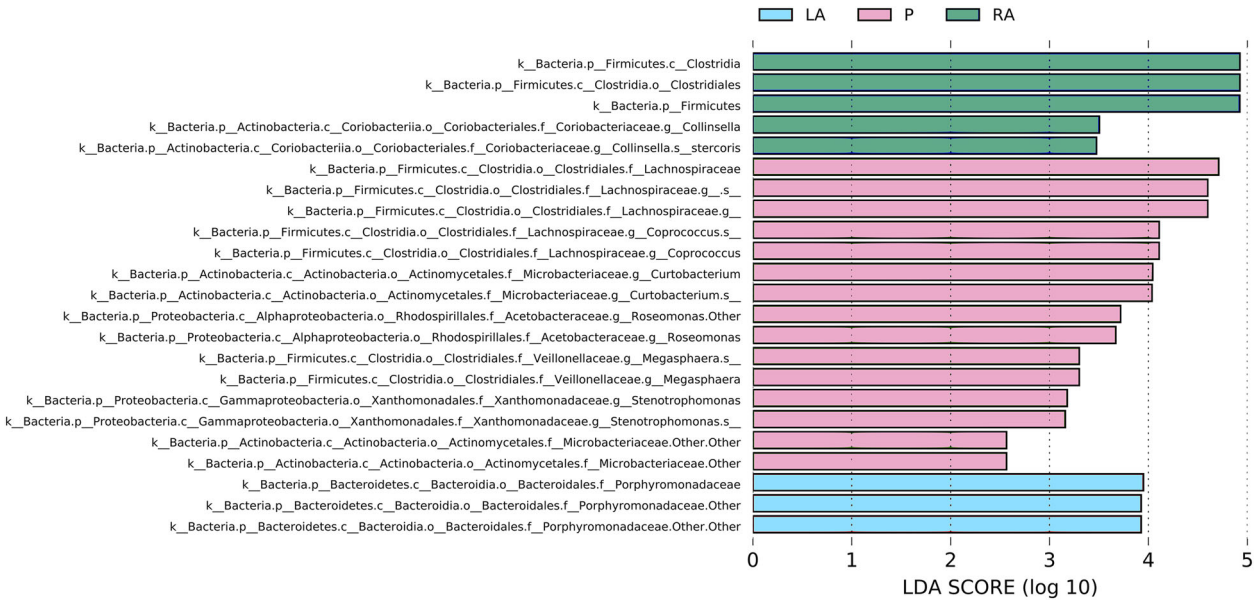

FIGURE 4 LDA scores for overrepresented taxa in each reproductive state based on LEfSe results

lactating females compared with both pregnant and cycling females ($z = -2.498$, adjusted $p = 0.033$; and $z = -2.987$, adjusted $p = 0.008$). Linear discriminant analyses showed that steroid biosynthesis and mineral absorption pathways were overrepresented in cycling females, cysteine, and methionine metabolism pathways—two pathways related to amino acid metabolism—were overrepresented in pregnant females, and riboflavin metabolism pathways were overrepresented in lactating females (Figure 6).

4 | DISCUSSION

This study provides the first evidence that the microbiome may play a role in supporting the energetic strategies of female primates during reproduction. As hypothesized, reproductive state was related to changes in gut microbial community composition and predicted function in this group of *C. capucinus*. However, our specific predictions regarding individual taxa and functional groups of microbes were not supported. Therefore, while the gut microbiomes of female *C. capucinus* in different reproductive states (cycling, pregnant, lactating) were distinct, additional research is necessary to confirm how changes in the gut microbiome between reproductive states influence the metabolic function and energy harvest potential of the gut microbiota.

Nevertheless, the patterns we observed provide an important foundation from which further studies can be developed. For example, when examining the specific taxa that characterize the gut microbiome in each reproductive state, we found an overrepresentation of Lachnospiraceae (Firmicutes) in pregnant females compared with cycling and lactating females. Pregnant women also have higher relative abundances of Lachnospiraceae (Firmicutes) in the first trimester (Koren et al., 2012), suggesting a potential role for this taxon. However, these results contrast with those from *A. pigra* that show that the relative abundance of Lachnospiraceae is positively correlated with dietary energy intake (Amato et al., 2015). Additionally, we did not find a reduction in the relative abundance of Bacteroidetes as energy requirements increased in response to reproductive state, as has been seen in pregnant humans (Collado, Isolauri, Laitinen, & Salminen, 2008). Instead, we found that the most energetically expensive reproductive state, lactation, was characterized by Porphyromonadaceae (Bacteroidetes).

We also detected an overall stability of the ratio of Firmicutes to Bacteroidetes across reproductive states, a result that is surprising given several studies of mammals that have shown that the ratio of

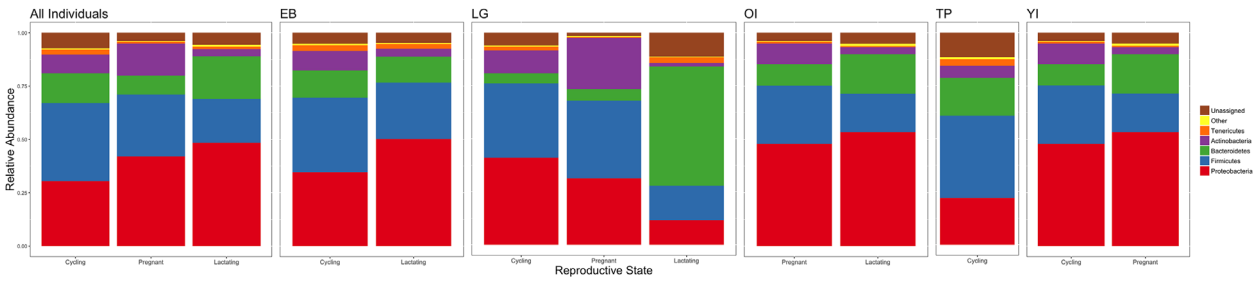

FIGURE 5 Relative abundance of bacterial phyla in the gut microbiota of *C. capucinus* individuals across reproductive states

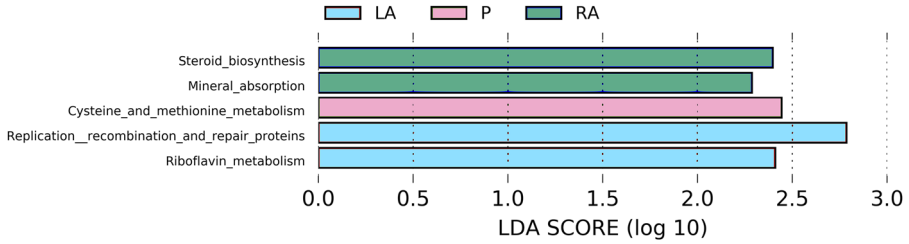

FIGURE 6 LDA scores for overrepresented predicted metabolic functions in each reproductive state based on LEfSe results

Firmicutes to Bacteroidetes increases in response to higher energy requirements or lower energy intake (Amato et al., 2014; Chevalier et al., 2015; Sonnenburg & Sonnenburg, 2014; Turnbaugh et al., 2009). However, measuring potential or actual rates of short chain fatty acid production is more informative for understanding the microbial contributions to host energy balances than the ratio of Firmicutes to Bacteroidetes (Duncan et al., 2008; Schwiertz et al., 2010). Therefore, in our dataset, short chain fatty acid production may vary between reproductive states independently of changes in the ratio of Firmicutes to Bacteroidetes.

The patterns we observed may be indicative of another mechanism for regulating the energy harvest potential of the gut. The increased relative abundance of Proteobacteria during pregnancy and lactation, as well as the significantly higher relative abundance of Actinobacteria during pregnancy, are similar to what has been reported in humans (Collado et al., 2008; Koren et al., 2012), suggesting an important role for these phyla during nutritionally demanding time periods. In fact, increased relative abundances of Proteobacteria have also been associated with periods of energetic stress in *Macaca thibetana* (Sun et al., 2016). Increases in Proteobacteria were also associated with a high fat diet in mice (Hildebrandt et al., 2009), indicating that Proteobacteria may be associated with host lipid metabolism. Capuchins rely heavily on insects in their diet, which are a high-fat, high-protein food resource (Raubenheimer & Rothman, 2013), and several other insect-feeding mammals have gut microbiota that are enriched in Proteobacteria (Bo et al., 2010; Delsuc et al., 2014; Xu et al., 2013). Additionally, data from captive *Sapajus apella* indicate that capuchin milk is higher in lipid content than that of either humans or closely related *Saimiri boliviensis* (Milligan, 2010), suggesting that lipid metabolism and uptake is critical for capuchins during lactation. As a result, we hypothesize that differences in the relative abundances of Proteobacteria in female capuchins during different reproductive states may facilitate increased energy uptake from lipid-rich food sources such as insects.

However, despite these associations, the beneficial role of Proteobacteria in the mammalian gut remains unclear. Increased relative abundances of Proteobacteria have also been associated with high rates of inflammation, low gut bacterial diversity, and type two diabetes in mice and humans (Bäckhed, 2011; Bäumler & Sperandio, 2016; Flint, Scott, Louis, & Duncan, 2012; Larsen et al., 2010; Morton et al., 2015). Many taxa within Proteobacteria are also potentially pathogenic in humans, including *Stenotrophomonas* and *Roseomonas*, genera that characterized pregnant females in this study (Dé, Rolston, & Han, 2004; Flint et al., 2012; Looney, Narita, & Mühlemann, 2009). Nevertheless, it is unlikely that high relative abundances of Proteobacteria in all of the seemingly healthy white-faced capuchin individuals in this study were a symptom of a systemic infection or sustained high rates of inflammation.

Finally, using predicted metagenome data, we observed changes in the predicted relative abundance of functional genes related to several metabolic pathways, including glycan metabolism, energy metabolism, cysteine and methionine metabolism, and riboflavin metabolism, between different reproductive states. This finding is distinct from what has been observed in humans (Koren et al., 2012), but again signals an important role of the gut microbiome in capuchin reproductive nutrition. For instance, increased riboflavin metabolism is likely related to increased nutrient needs during lactation (Allen, 2012; Hampel et al., 2016). However, our data describing potential functional differences in the gut microbiome between reproductive states should be interpreted cautiously since the capuchin microbiome is poorly characterized. In particular, additional studies of the functional role of Proteobacteria in the gut microbiota of *C. capucinus* are warranted, as the relationship between Proteobacteria and metabolic functions and other functional roles in this host species are unknown.

While the results of this study indicate an interaction between reproductive state and the gut microbiota of *C. capucinus* in this population, small sample size as well as the limitations of our understanding of the interactions between the environment, host physiology, gut microbial community structure, and gut microbial community function in wild primates make interpretation difficult. These results, nevertheless, suggest the gut microbiome may play a role in the energetic strategies of *C. capucinus* females during reproduction, particularly strategies related to the high energetic demands of accelerated post-natal brain growth during lactation (Elias, 1977; Hartwig, 1996; Phillips & Sherwood, 2008).

Additionally, the results challenge the expected relationships between increases in energy expenditure and changes in gut microbial community structure, highlight the complexity of host-microbe relationships, and underscore the importance of interspecific differences in host-microbe relationships. Since reproductive state accounts for less than 10% of the variation in the composition of the gut microbiome, other factors such as behavioral and dietary adaptations are undoubtedly playing a role in how *C. capucinus* females are meeting their changing energetic demands. However, time spent feeding and foraging on fruit or invertebrates was not related to changes in gut microbial community composition in this population, nor did female reproductive state covary with diet composition during the study

period. It is likely that changes in hormones associated with pregnancy and lactation and/or variation in energy or macronutrient intake are primarily contributing to the changes in gut microbiome between reproductive states. Future studies of reproduction-associated changes in the gut microbiome that incorporate measures of reproductive hormones and nutrient intake are warranted. Moving forward, the integration of data describing the structure and function of the gut microbiota in studies of the reproductive ecology of primates will help refine to what extent the gut microbiome interacts with and is influenced by the reproductive strategies of primates. These studies of the "microbial reproductive ecology" of primates will help elucidate how microbial contributions to primate energetic strategies may have influenced the evolution of expanded brain size, accelerated post-natal brain growth, and prolonged periods of infant dependency in primates.

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CONFLICTS OF INTEREST

The authors do not have any conflict of interest to declare.

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