Integrative Zoology 2022; 17: 1147–1161 doi: 10.1111/1749-4877.12601

ORIGINAL ARTICLE

Seasonal diet and microbiome shifts in wild rhesus macaques are better correlated at the level of nutrient components than food items

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Abstract

Food supply is one of the major drivers of animal behavior, and the gut microbiome is an important mediator between food supply and its effects on physiology. However, predicting the outcome of diet change on microbiome and consequences for the animal has proven extremely challenging. We propose this reflects processes occurring at different scales. Inadequate accounting for the multi-level complexity of nutrition (nutrients, foods, diets) obscures the diet influence on microbiome and subsequently animal. Here, we present a detailed year-round, multi-level analysis of diet and microbiome changes in a wild population of a temperate primate, the rhesus macaque (*Macaca mulatta*). Total daily food and nutrient intake of 6 male and 6 female macaques was monitored in each of the 4 seasons (total 120 days observations). For each individual, we found significant variation in the microbiome between all 4 seasons. This response was more strongly correlated with changes in macronutrient intake than with food items and much of the response could be explained at the level of 6 ecological guilds—sets of taxa sharing similar responses to nutrient intake. We conclude that study of diet, microbiome, and animal performance in ecology will more effectively identify patterns if diet is recorded at the level of nutrient intake. Although microbiome response to diet does show variation in species-level taxa in response to food items, there is greater commonality in response at the level of guilds. A goal for microbiome researchers should be to identify genes encoding microbial attributes that can define such guilds.

Key words: gut microbiome, macaque, nutrition, nutritional ecology, seasonality

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INTRODUCTION

It is now generally accepted that the microbial community of the animal gut (henceforth gut microbiome) profoundly influences many aspects of animal physiology and well-being (Sommer & Bckhed 2013). The range of known or postulated microbiome impacts in humans and other animals encompasses so many aspects of behavior, physiology, and development that we might expect adaptive regulation of the host–microbiome relationship to be a significant factor for evolutionary fitness (Amato *et al*. 2019b; Björk *et al*. 2019; Muegge *et al*. 2011). Thus, understanding how an animal gut microbiome responds to variation in the environment, and how this response varies between individuals of the same species, is fundamental to understanding animal biology (Amato *et al*. 2019a; Gomez *et al*. 2019).

A major environmental factor in regard to animal health and microbiome dynamics is diet. The availability and the nutrient content of food items vary in both time and space. For example, seasonal variation in temperature, rainfall, and day length influence both plant foods (leaf/fruit nutrient content) and foraging hours. Collectively, such seasonal or geographic variation will influence the absolute amount, relative availability of, and intake periodicity of food-derived nutrients to the host animal and its gut microbes. These diet dimensions are all known to impact animal physiology as well as microbiome properties (Wu *et al*. 2011; David *et al*. 2014; Uhr *et al*. 2019). This gives rise to questions as to how members of the same animal species (and their microbiomes) adapt to such nutritional transitions through behavioral, physiological, or microbial mechanisms and the relative importance of different dimensions of diet in driving responses (Amato *et al*. 2015; Gomez *et al*. 2016; Koch *et al*. 2017; Springer *et al*. 2017; Trosvik *et al*. 2018b). There are, however, several challenges for addressing this question.

A primary challenge for understanding how animals adapt to ecologically imposed resource transitions is to identify the relevant dimensions to characterize resources. Much previous work in animal ecology has focused on characterizing diet by food type (e.g. leaf, seed, or fruit dominance). However, foods are complex mixtures of many functionally relevant components, and these are compounded during foraging into higher level mixtures, such as meals and ultimately diets. Nutritional geometry (NG) provides a framework to characterize resource intake across the multiple levels of resource acquisition, from individual nutrients, nutrient mixtures, and foods (Raubenheimer *et al*. 2009; Simpson & Raubenheimer 2012). Many NG studies have demonstrated that characterizing resources at the appropriate level is critically important for understanding the dietary strategies of animals, because the different levels can lead to different, sometimes opposing, conclusions. For example, primates typically experience substantial variation in the relative availability of high protein foods (e.g. leaves) versus foods with high fats and carbohydrates (e.g. seeds and fruits). In several species, however, this variation does not translate into variation in protein intake because they regulate macronutrient intake such that absolute protein intake is maintained constant in the face of variation in the protein:non-protein ratio of the diet, while intake of fats and carbohydrates varies (Felton *et al*. 2009). Other studies have shown that species can be dietary generalist at the level of foods eaten, yet specialists at the level of nutrient intake (Cui *et al*. 2018, 2019). Finally, a recent study showed that despite being highly specialized herbivores, at the macronutritional level, giant pandas resemble carnivores in eating a diet with over 50% of energy contributed by protein (Nie *et al*. 2015). This insight might explain why giant pandas have retained several diet-related adaptations that resemble carnivores, including gut structure, digestive enzymes, and the gut microbiome (Wei *et al*. 2015).

For microbiome response, an additional challenge is to disentangle diet-driven variation from the many other factors that determine the microbiome of individuals. There is abundant evidence that factors unique to an individual animal are important constraints on microbiome response to diet change. For example, Wu *et al*. (2011) conducted controlled feeding experiments with humans such that intake of the macronutrients fat and carbohydrate varied. They found that the post-diet microbiome outcomes were more strongly determined by the individual subject than the diet manipulation (Wu *et al*. 2011). Further insight comes from David *et al*. (2014) who monitored the temporal dynamics of human microbiomes over diet transitions. They found an individual's microbiome was resistant to change in response to transition to a plant-dominated diet (with reduced intake of fat and protein and higher intake of fiber) but showed significant change in response to animal-dominated diet (with increased protein and fat intake and almost no fiber intake). However, upon return to the baseline diet, the microbiome returned to the prior state indicating individual microbiomes show resilience to diet disturbance (David *et al*. 2014). These, and other, studies show that the microbiome is resistant to change within limits but that major changes in intake, especially of fiber, will drive microbiome change. Further, as has repeatedly been found, neither the responsive taxa nor the physiological consequences are consistent across all individuals (Walker *et al*. 2011;

Martens *et al*. 2014; Kovatcheva-Datchary *et al*. 2015; Smits *et al*. 2016; Makki *et al*. 2018). Collectively, these observations in humans show that microbiome response to diet variation is constrained by factors that are related to an individual. Since individual, or social, group effects have also been observed in microbiomes of wild primate populations experiencing diet variation (Bennett *et al*. 2016; Amato *et al*. 2017; Springer *et al*. 2017; Trosvik *et al*. 2018a; Orkin *et al*. 2019), it is probable that similar mechanisms constraining the microbiome response to nutrient stresses imposed by diet are present in most primates. Microbiome response to seasonal diet variation is thus constrained at the level of the individual. In assessing the ecological significance for animal species, we need to look for microbiome patterns that are consistent between individuals.

NG designs have also been used in laboratory studies to disentangle the effects of nutrient dimensions and energy intake on the gut microbiome and associated responses of the animal hosts. We recently showed in laboratory mice that the relative availability of dietary or endogenous sources of nitrogen underpins changes in microbiome structure in response to total calorie intake (Holmes *et al*. 2017). This occurs owing to distinct microbial foraging strategies for endogenous versus dietary nitrogen sources that favor different guilds under conditions of low and high intake. We postulated that because this mechanism has the potential to operate consistently across multiple diet dimensions, including those where fasting periods vary (e.g. with day length), where total energy availability is altered (e.g. famine) and across variation in the distribution of protein and non-protein energy sources, it would be of broad significance to animal biology (Holmes *et al*. 2017). No study, however, has attempted to achieve this in the ecologically relevant context of wild animals confronted by natural variation in energy or protein availability.

In this study, we used NG to examine individual and population responses of the gut microbiome diet transitions in a wild population of macaques (*M. mulatta tcheliensis*) in a temperate climate experiencing strong seasonal variation in foods with respect to energy density and nutrient balance. The animals have been habituated to human observation such that collecting high-resolution data on food and nutrient intakes of focal individuals over prolonged periods is possible. By pairing 120 full-day dietary observations with analyses of individual-matched fecal samples collected across the annual cycle, we were able to examine gut microbiome responses to seasonal dietary shifts in foods and nutrients eaten and determine between-individual variation in these responses. All 4 seasonal diets have potential to drive microbiome change in individuals, but we predicted that the mechanisms involved and their relative strength would likely differ. Based on the demonstration in previous research that food selection in our study population (and many other primate species) is driven strongly by macronutrient balancing (Johnson *et al*. 2013; Cui *et al*. 2018; Hou *et al*. 2021), we predicted that seasonal variation in macronutrients would explain the major portion of variation in the gut microbe. Specifically, based on our rodent models, we hypothesized that if seasonal patterns in microbiome were shared across individuals, they would most likely be driven by the generic mechanism of interaction between dietary and endogenous protein sources with total energy intake.

MATERIAL AND METHODS

Sample collection in the field

All field observations and specimens were from a single troop of rhesus macaques (*Macaca mulatta tcheliensis*) that had become habituated to human observation over 4 years. The study site is temperate deciduous forest with 4 seasons. In order to obtain matched datasets for seasonal diet composition (nutrient intake) and microbiota, 12 individuals were intensively followed across the seasonal year June 2016 to May 2017. These comprised 6 adult males and 6 adult females. To limit the impact of pregnancy or lactation on nutritional demands, we included females that did not have dependent offspring at the study commencement. The selected individuals also did not give birth to infants in the spring 2017 season. Details of feeding observation methodology and nutritional content of food items have previously been reported (Cui *et al*. 2018, 2019), and key aspects are repeated below. Body mass (summer, means ± SE males = 8.5 ± 0.5 kg, females = 6.7 ± 0.02 kg; autumn, males = 9.9 ± 0.5 kg, females = 8.2 ± 0.3 kg; winter, males = 9.2 ± 0.4 kg, females = 6.9 ± 0.2 kg; spring, males = 8.7 ± 0.5 kg, females = 7.1 ± 0.1 kg. Details on the bodyweight can be found in Table S2, Supporting Information) was determined non-invasively for all animals across 4 seasons, using small amounts of corn to coax the monkeys to step onto an electronic scale (Qianxuan TS-2010A4, accuracy 0.1 kg; Zhang *et al*. 2016).

Diet data: food item composition

The 12 focal individuals were followed from dawn to dusk according to strict criteria of

observational continuity. Data were only used for days where the individual animal was maintained under continuous observation to ensure a complete record of daily food intake (see Cui *et al*. 2018 for details). The 12 focal individuals for this study were followed through 4 separate field observation periods, representing a 2 month window for each season. For each individual animal a minimum of 2 feeding observation days per season was collected and across the study group of 12 animals we accrued a total of 120 whole days of observations.

Diet data: nutrient intake determination

Representative samples of all major food items were collected in each season and nutrient intakes determined as described in (Cui *et al*. 2018). Briefly, food nutrient compositions were multiplied by the amount of each food eaten within the observation period, and intakes expressed as available protein, fat, and carbohydrates. Carbohydrates were partitioned into the major categories, namely total non-structural carbohydrate (TNC—including starch and soluble sugars) and neutral detergent fiber (NDF—including cell wall compounds such as hemicellulose, cellulose, and lignin).

Crude protein was determined by the Kjehdahl assay (BUICHI, KjelFIex K-360; Brooks *et al*. 1995; Nioi *et al*. 2012) with 6.25 as the conversion factor, and available protein (AP) was estimated through subtraction of acid detergent insoluble nitrogen from crude protein (Licitra *et al*. 1996). Fat was measured with a fat analyzer (FOSS, SCINOTMST310) using the ether extraction method (Rothman *et al*. 2012). Samples were analyzed for NDF with residual ash (with sodium sulfite and a-amylase), then for acid detergent lignin (ADL) and acid detergent fiber (ADF) using an automatic fiber analyzer (ANKOM, A2000i; Goering & Van Soest 1970; Van Soest *et al*. 1991; Rothman *et al*. 2008), and total nonstructural carbohydrate (TNC) was calculated by difference (Johnson *et al*. 2013). Available energy from fiber was estimated based on previously published assumptions for the NDF digestive efficiency (Edwards & Ullrey 1999; Rothman *et al*. 2012), and the energy contribution from NDF calculated by multiplying the conversion values of 16.7 kJ per g TNC by the NDF digestibility coefficients, and then subtracting the 4 kJ per g estimated to be consumed by gut microbes (Conklin-Brittain *et al*. 2006; Rothman *et al*. 2008; Cui *et al*. 2018). For macronutrients, we used the conventional conversion values of 37.7 kJ/g crude fat, 16.7 kJ/g AP, 16.7 kJ/g TNC (NRC 1989), and 6.8kJ/g NDF (Cui *et al*. 2018).

Fecal sample collection

Feces were sampled in summer (3 Jun—6 July, 2016), in autumn (3 Sept—14 Oct, 2016), in winter (1 Jan—2 Jan, 2017), and in spring (1 Mar—26 May, 2017) giving a total of 48 fecal samples. To the greatest extent possible, fecal samples were contemporaneous with diet observations of the same individual, or other individuals in the same feeding group. Fecal samples were all taken where the identity of the animal was noted at defecation and the sample collected from the ground within minutes of deposit. To minimize environmental contamination, we physically broke apart the stools to collect only the inner part as the fecal sample. All fresh fecal samples were collected into sterile 40 ml containers, and then placed in a tube of RNAlater immediately. Samples were subsequently stored at −80°C until DNA extraction. However, there are constraints on ensuring natural feeding behavior and fecal donor identity that mean that it was seldom possible to have diet observations and fecal sample collection made on the same day. Fig. 1 summarizes the temporal pattern of feeding observations and stool collection throughout the year. Full data and the accession number of sequences are available in Table S1, Supporting Information. For analysis of association between diet intake and microbiome, we used the seasonal averages of intake of the individual (Table S1, Supporting Information).

DNA extraction and sequencing

Total DNA was extracted from samples using a QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD, USA). Extracted DNA was sent to TinyGene Bio-Tech Co., Ltd., Shanghai, China, for sequencing. The primers 515F 5′-GTGCCAGCMGCCGCGGTAA-3′ and 926R 5′-CCGTCAATTCMTTTGAGTTT-3′ were used to amplify the V4-V5 region of the 16S rRNA gene (Caporaso *et al*. 2012; Langille *et al*. 2013; Hill *et al*. 2017). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2× 300 bp sequencing was performed using NGS Illumina MiSeq platform. Sequencing service was provided by TinyGene Bio-Tech (Shanghai) Co., Ltd.

Sequence analysis and data analysis

The raw fastq formatted files were demultiplexed based on the barcode. Pair-end reads for all samples were trimmed through Trimmomatic (version 0.35) to remove low quality base pairs using parameters (SLIDINGWINDOW: 50:20 MINLEN: 50). Trimmed reads

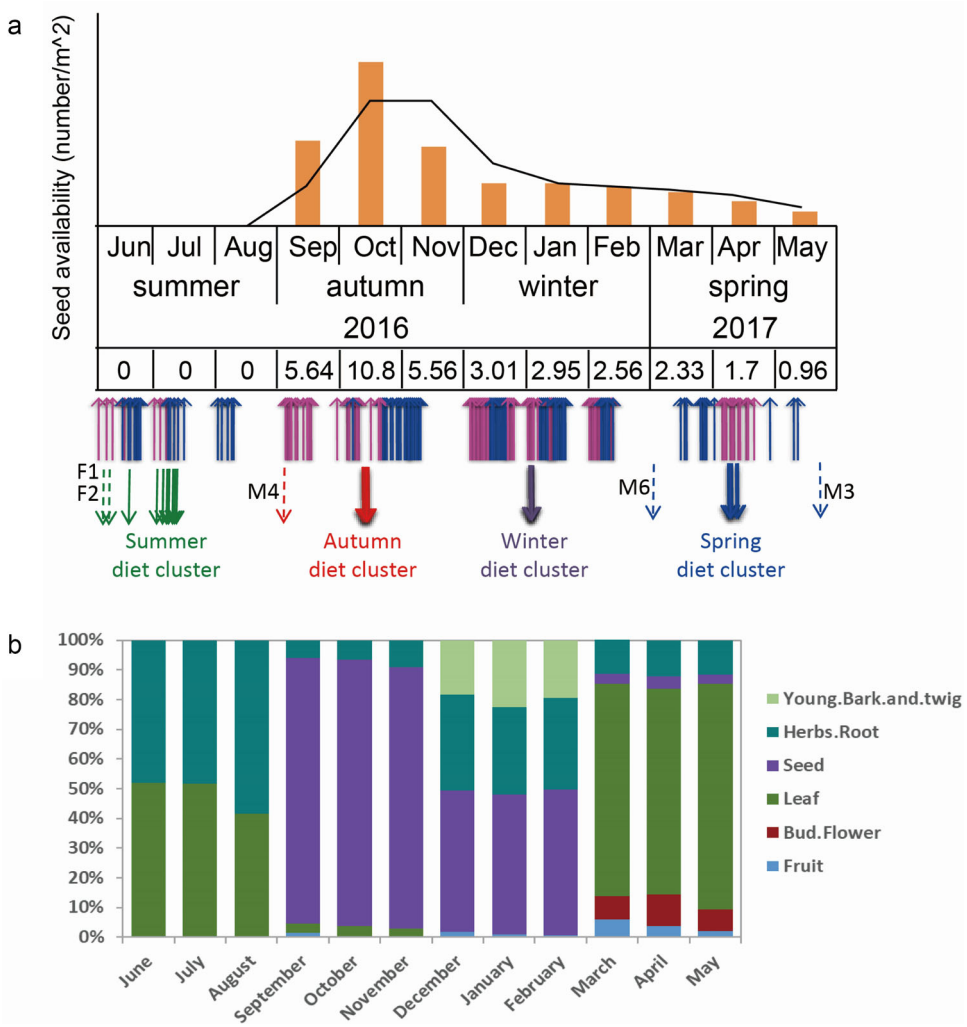

Figure 1 Relationship between food availability, diet observations, and fecal sample collection. (a) Temporal relationship between acorn availability, food intake observations, and collection of fecal samples. Food observations are shown by up arrows (blue-males and pink-females). Down arrows show time points for fecal samples colored according to their assigned season, dashed lines are times closer to seasonal boundaries. (b) Average diet composition by month. Data are the average diet composition from all available observations in that month of the same 12 focal individuals.

were then further merged using FLASH (version 1.2.11) with default parameters. The low quality contigs were removed using screen.seqs command following the filtering parameters, maxambig = 0, minlength = 200, maxlength = 580, maxhomop = 8. The 16S sequences were analyzed using a combination of software mothur (version 1.33.3), usearch (version v8.1.1756, http://drive5.com/uparse/), and R (version 3.2.3). The demultiplexed reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) following the UPARSE pipeline (http://drive5.com/usearch/manual/uparsecmds.html), and the OTU representative sequences were assigned for taxonomy against Silva 119 database with confidence score $\geq$ 0.8 by the classify.seqs command in mothur. OTU taxonomies (from Phylum to Species) were determined based on NCBI. For the alpha-diversity analysis, Shannon, Inverse Simpson, Chao1, ACE index, and rarefaction curves were calculated using mothur and displayed using R.

For the beta-diversity analysis, the weighted and unweighted UniFrac distance matrix were calculated using mothur and visualized by principal coordinate analysis (PCoA) using R packages "GUniFrac" and "ape". To analyze the effects of food and nutrient intakes, we used

R packages "vegan" analysis distance-based redundancy analysis (db-RDA) with input beta diversity distance matrix and Root, Leaf, Fruit, Seed, AP, F, TNC, NDF, and Male factors. We analyze the overall difference of environmental factors separately by ANOVA.

We used R packages "Spearman" to calculate the correlation coefficient between nutrition, food types, and microbial OUT abundances, and "PHEATMAP" to perform cluster analysis on the correlation coefficient.

RESULTS

Seasonal nature of the stool and diet data sets

We collected stool samples from identified individuals that were contemporaneous with diet observations and focused on temporal windows that lay midway within each season. This gave 4 series of seasonal food intake data, each separated from the adjacent seasonal series by a minimum of 3 weeks. With 5 exceptions, stool samples were taken at time points in the middle of the seasonal observation series. The relationship between diet observation, stool samples, and time is summarized in Fig. 1a.

Seasonal variation in food availability, diet composition, and nutrient intake

We found marked food item differences between seasons and only limited variation between individuals within a season. Fig. 1b shows a summary of the major categories of food items in the diet across all sampled individuals for the month. The sharpest transitions are: summer–autumn, reflecting the defined onset of availability of acorns in late august and winter–spring, reflecting the combination of depletion of acorns and sharp onset of new plant growth and flowering (see also Cui *et al*. 2019 for more detail). This pattern indicates that within a seasonal data series, composition of macaque diets is expected to be broadly consistent across all 12 focal individuals. However, sharp differences are expected between the seasons since phytochemical composition is known to vary across the dominant plant food items (e.g. ripe vs unripe acorns and tannins in ripe acorns are far higher than in herbs).

Our nutrient intake data is at the level of the individual animal per day. Food item composition data in each season was used to parse the data for an individual's daily food intake into 4 macronutrient dimensions of protein, fat, neutral detergent fiber, and total non-structural carbohydrates. We further simplified this to 2 dimensions of available protein (AP) or non-protein (NP) energy intake, and expressed as kJ per kilogram body weight per day (kJ/BWkg/day). By this nutrient-based analysis, the 4 inter-seasonal distinctions are not so strongly defined as seen for the item-based analysis (Figs 1b, 2). In nutrient space, the autumn/winter diets form a cluster that is sharply distinguished from spring/summer diets by the ratio of energy intake as NP to that as AP (>5:1 vs <3:1). This is primarily driven by the strong targeting of the NP dense acorns (*Quercus* spp.) when they are abundantly available (autumn and winter) versus increasing utilization of leaf and herb items that have lower energy density and density NP:AP when they are not (Fig. S1b, Supporting Information). Nevertheless, there are significant differences between each of the 4 seasons with respect to nutrient intake. Autumn and winter were significantly different with respect to AP intake (mean of 54 kJ/BWkg/day vs 45 kJ/BWkg/day; $P < 0.001$) and NP intake (mean of 359 kJ/BWkg/day vs 340 kJ/BWkg/day; $P < 0.05$). We note the form of non-protein energy was also different with autumn having significantly higher fat intake (Fig. S1, Supporting Information). Spring and summer were more markedly distinct from each other with all summer samples having NP:AP <2.5:1. Thus, the summer diet was the outlier in nutritional terms with significantly higher proportion of protein in the diet and lower total energy intake than the other 3 seasons (Fig. 2). We conclude that for each of the 4 seasons the differences in macaque diets have the potential to drive microbiome (or host) changes and that the resource limiting factors may vary between seasons.

Composition of the macaque gut microbiota

For 12 individuals in each season, microbiome was profiled by amplicon sequencing of the V4-V5 region of the bacterial ribosomal RNA. We obtained a total 1 994 971 reads after quality filtering (ave 41 561 ± 7125; range 23 358 to 51 188 reads per sample). OTUs were picked at 97% identity and taxonomic assignment revealed the microbiota was typical of the primate gut, being largely dominated by Firmicutes (56.14% ± 6.46%) and Bacteroidetes (36.57% ± 6.80%). Other represented phyla were Tenericutes (2.44% ± 2.05%), Spirochaetes (2.00% ± 1.29%), Proteobacteria (1.43% ± 1.29%), Actinobacteria (1.15% ± 1.25%), Fibrobacteres (0.09% ± 0.12%), and Cyanobacteria (0.05% ± 0.06%). The predominant genera were *Prevotella* (17.67% ± 8.62%), *Treponema* (1.73% ± 1.31%), and *Faecalibacterium* (1.45% ± 1.33%). Using the Shannon index as a basic measure of alpha diversity, we found significantly lower diversity in summer compared to the other 3 seasons (Fig. 3a). Spring also had lower mean Shannon

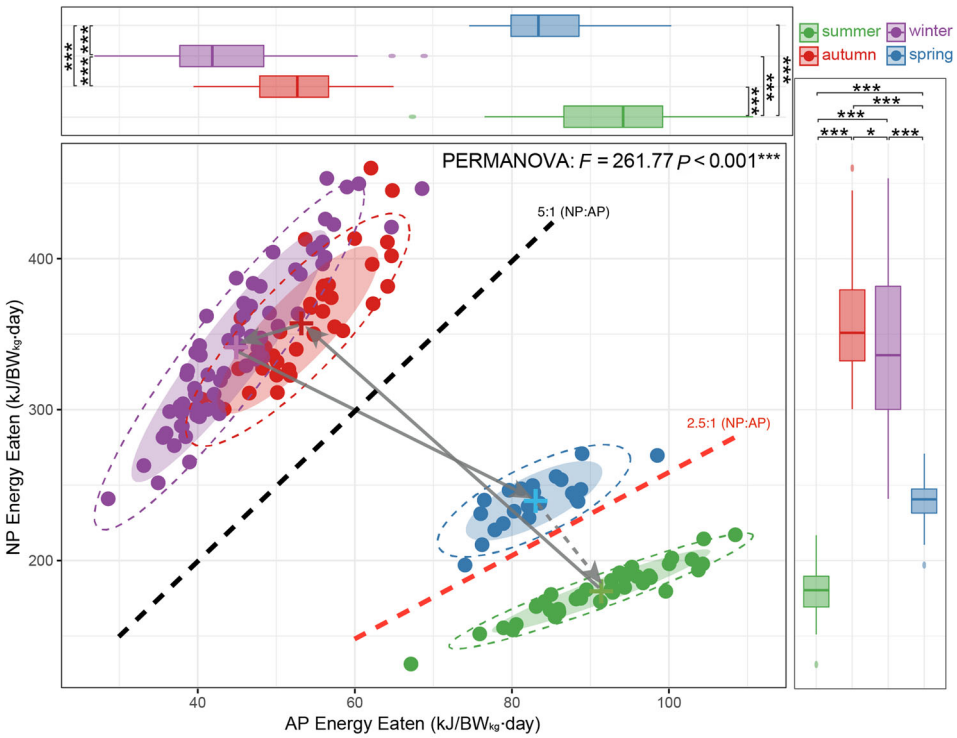

Figure 2 Seasonal pattern of nutrient intake. Each dot represents one day observation of one individual. There is a distinct separation of the acorn-dominated (autumn/winter) and leaf-dominated (spring/summer) seasons. When diet intake is parsed into all protein and non-protein (sum of Fat, NDF, and TNC), all 4 seasons show significant differences in nutrient intake by PERMANOVA ($^{*}P < 0.05$; $^{***}P < 0.001$).

diversity than autumn and winter, but the difference was not significant ($P > 0.05$).

Gut microbial communities show seasonal patterns

Principle co-ordinate analysis showed that each individual's community structure varied over time and that the communities showed a stronger pattern of relationship by season than source animal (Fig. 3). This indicates that, across this macaque population, factors intrinsic to the microbiome of an individual animal do not obscure annual patterns of microbiome community structure due to seasonal variation in the diet. In PCoA using unweighted Unifrac distances, evidence for significant differences between all 4 seasons was seen (PERMANOVA; $P < 0.001$), with the greatest differences being between summer and all other samples (Fig. 3b). Similar patterns were seen in NMDS analysis (data not shown).

In this analysis, there are 5 samples that show aberrant relationship to other samples from the same season (Fig. 3b). These are F1_Su, F2_Su, M4_Au, M3_Sp, and M6_Sp. Our diet observations did not show these individuals as outliers for each sample season (Fig. 2); however, those 5 stool samples are also the only ones that were not collected in the mid-period of their nominal sampling seasons (Fig 1a). Sample collection constraints meant they were collected nearer the transitions of spring–summer (F1_Su, F2_Su, and M3_Sp), summer–autumn (M4_Au), or winter–spring (M6_Sp), where there is less information on the individuals' recent diet history. For example, in the case of F1 and F2, although we have contemporaneous diet observations and stool samples for early June 2016, there are no diet data for the focal animals in the preceding 3 weeks of May. Thus, we cannot rule out the possibility that seasonal diet attribution for those individuals' sample points was inaccurate.

To explore the explanatory value of food items or nutrients for microbiome structure, we conducted DB-RDA. This analysis showed that both had comparable levels of explanatory power, but that nutrients best explains the isolation of summer from the autumn, winter, spring clusters and the separation of autumn from winter (Fig. 3c), whereas food items better explained the separation of spring from autumn/winter (Fig. 3d). This

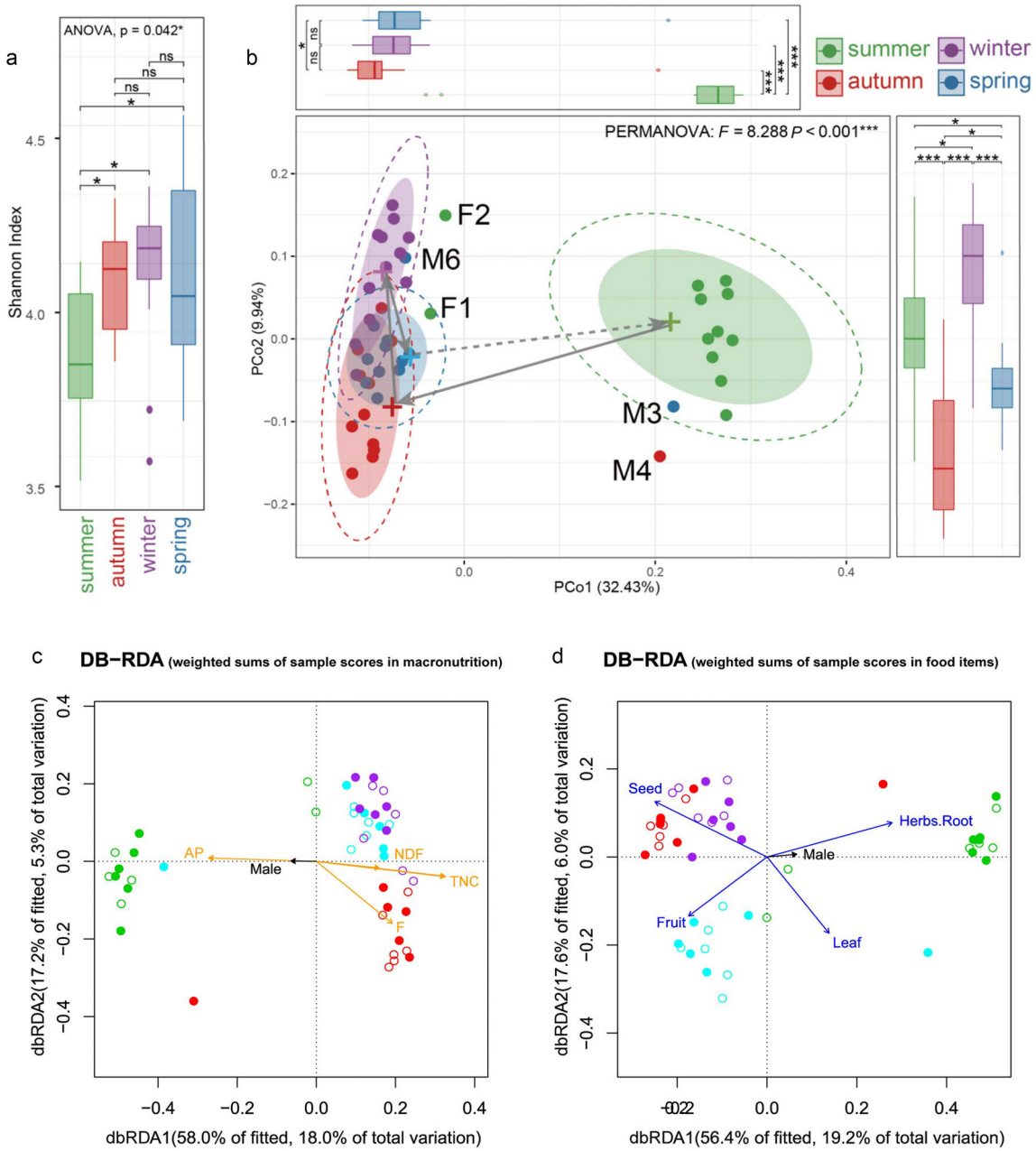

Figure 3 Seasonal patterns of microbial diversity. (a) Summer has significantly lower Shannon diversity than the other 3 seasons. (b) PCoA of unweighted unifrac distances colored by season. All 4 seasons are significantly different, with summer as a distinctive outlier. Note the samples from F1, F2, M3, M4, and M6 were collected at seasonal transitions as shown in Fig. 1. The dbRDA ordination plot using unweighted UniFrac distance with nutrition, food, and sex variables. (c) Weighted sums of samples scores in macronutrition; (d) weighted sums of samples scores in food. Total or fitted variations refer to the variances of original unweighted UniFrac distance data or the constrained variance in the analysis. Samples collected in spring, summer, autumn, and winter are color-coded as cyan, green, red, and purple, respectively. Solid and hollow circles indicate samples of male and female hosts. Explanatory variables of nutrition, food items, and sex are marked as orange, blue, and black arrows. The DB-RDA model were uwUniFrac ~Leaf + Fruit + Seed + Herbs.Root + Male, and uwUniFrac ~AP + F + TNC + NDF + Male, and the test results of the model were significant difference, respectively (Fig. 3, C: $F = 4.3583$, $P = 0.001$, DB-RDA1:$F = 12.2785$, $P = 0.001$, DB-RDA2: $F = 3.8315$, $P = 0.001$; Fig. 3 D: $F = 3.7794$, $P = 0.001$, DB-RDA1: $F = 10.9607$, $P = 0.001$, DB-RDA2: $F = 3.2503$, $P = 0.002$).

is consistent with distinct types of food-related selective pressure on the observed microbiome response, one captured best by macronutrients and the other by food items.

More microbe taxa correlated with seasonal changes in macronutrient intake than with food items

To identify potential drivers of the community change, we looked for correlations between relative abundance changes in major taxa and average daily nutrient or food item intakes (detailed data on AP: available protein, NDF: neutral detergent fiber, F: fat, and TNC: total nonstructural carbohydrates are summarized in Table S2, Supporting Information). More correlations were found for nutrient intake than for food item categories, and on the basis of cluster analysis, we identified 6 putative guilds, sets of taxa that share similar responses to the same nutrient dimensions (Fig. 4). In broad terms, guilds g1 to g4 were typified by a negative correlation to AP intake and positive correlation to NP intake; guild 5 members showed positive correlation to AP intake and negative correlation to NP intake; and guild 6 members generally showed an absence of strong correlation to any dietary nutrient category.

The effect of seasonal changes in diet on representative members of these guilds is shown in Fig. S2, Supporting Information. For members of guilds 1 and 2, their maximal occurrence was strongly over-represented in the autumn and winter samples, and with the exception of *Lachnoclostridium*, abundance distribution for all taxa in these guilds showed a significant difference ($P < 0.01$) between summer and autumn samples. That seasonal transition is typified by a dramatic increase in the macaque utilization of acorns in the diet whereby the autumn animals have a large increase in non-protein energy relative to summer and a decrease in available protein. These 2 guilds are distinguished by their performance in summer (relative abundance of G2 members persists better than G1). Although the specific taxon showing the strongest response to the switch from summer to autumn diets varied from animal to animal, the response pattern was consistent across individuals. This distribution of the response signal across a set of phylogenetically distinct OTUs, rather than dominance by a single successful taxon, is consistent with the pattern reflecting common resource limitation effects shared by different species. The difference between spring and summer communities is due to members of G5 and G1 having low relative abundance on the summer diets. Collectively, these observations indicate that changes in food item (including carbohydrate type) and macronutrient intake both impact microbe responses, but the intake rate and distribution of macronutrients is more predictive of the fundamental drivers of summer as an outlier than food type (Fig. 3).

DISCUSSION

We present a detailed dataset of diet and microbiome composition in 12 macaques in a temperate habitat over a full year cycle. Much previous work in animal and gut microbial ecology has focused on simplifying diet to either food type (e.g. leaf, seed, or fruit dominance) or carbohydrate type (e.g. starch, xylan, cellulose), respectively. Here, food availability and quality differed strongly between all 4 seasons and we found describing diet in terms of nutrient intake was a better predictor of the major microbiome impact than the seasonally dominant food type. Our data support the hypothesis that a significant proportion of the complexity of diet × microbiome interaction can be simplified to macronutrient intake dimensions that capture the availability of protein and non-protein energy to both host and microbes, as previously demonstrated in laboratory experiments of mice (Holmes *et al*. 2017). A plausible physiological explanation for this is that most gut microbes have an extensive and overlapping repertoire of CAZymes to enable access to diverse carbohydrates as carbon and energy sources and a comparatively narrow range of enzymes to access nitrogen sources. Although individual taxa may specialize for utilization of different carbohydrate substrates and experience limitation as food items shift, at the whole community level, nitrogen limitation is more frequently encountered. Consequently, where seasonal transitions are characterized by big shifts in relative availability of nitrogen sources, strategies for adapting to this dominate the outcome and relative protein intake has high predictive value. We discuss ways in which this is a useful addition to the conceptual framework for nutritional ecology of microbes, of animals, and for understanding of nutrition-related chronic disease in humans.

Temporal changes in foraging behavior, diet, and microbiome have been documented in a number of primates including humans, apes, lemurs, and new and old world monkeys (Amato *et al*. 2015; Gomez *et al*. 2016; Springer *et al*. 2017; Hicks *et al*. 2018; Trosvik & de Muinck *et al*. 2018a; Orkin *et al*. 2019). A common finding of all studies is that season (or sampling time) was associated with change in beta-diversity patterns of microbiome. An obvious hypothesis to explain this is that seasonal change in

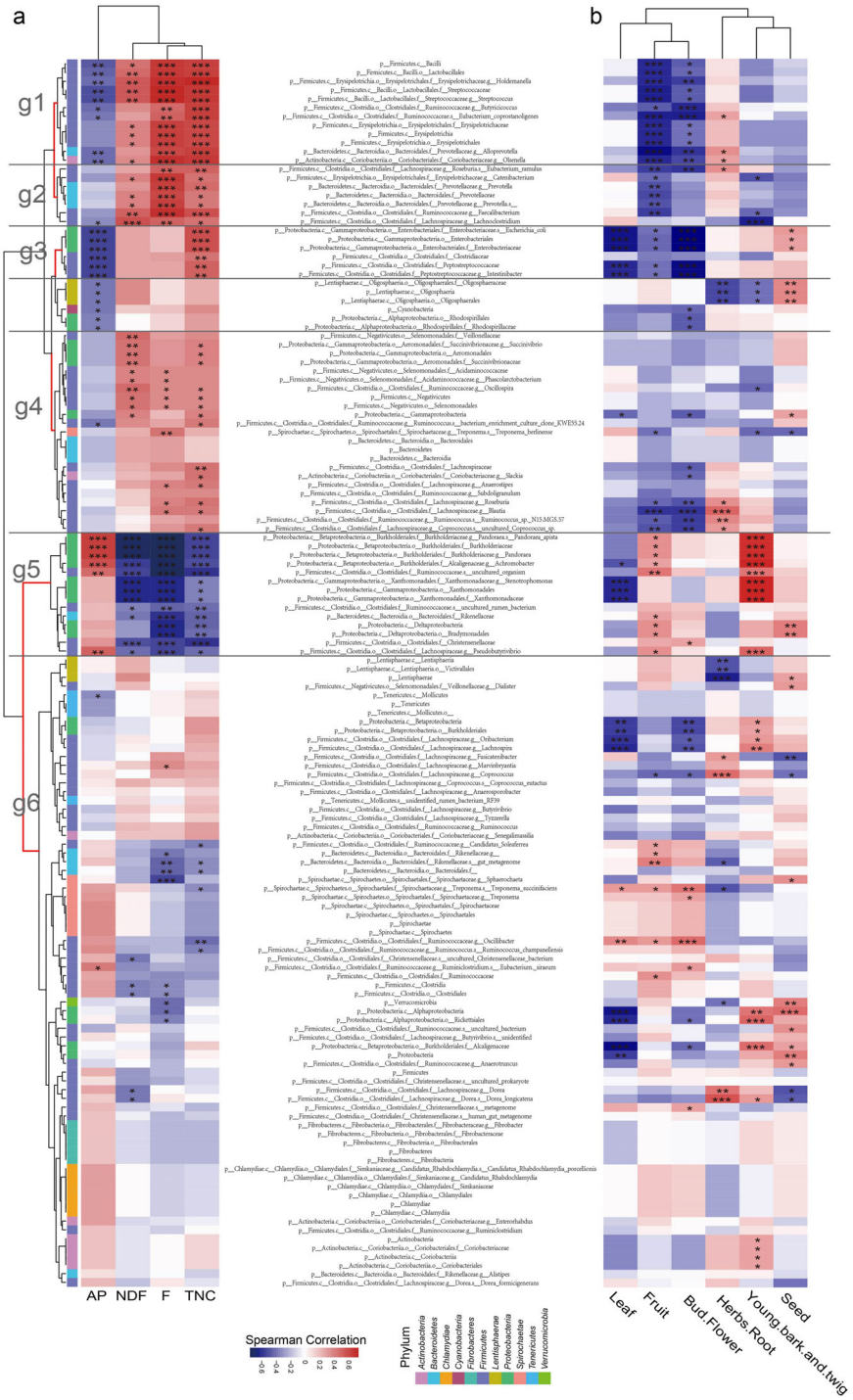

Figure 4 (a) Nutrient response guilds within the microbiome. Spearman correlation for taxon abundance with nutrient intake. (b) Food item response guilds within the microbiome. Spearman correlation for taxon abundance with nutrient/food intake. Six groups of taxa showing shared response patterns to nutrient intake and food item are identified. Phylum affiliations for all taxa are shown to the left and selected genera on the right.

diet drives the observed change in microbiome, but disentangling this from other variables and interpreting the physiological significance for the animal is difficult for 2 reasons. First, the majority of these studies have been in the tropics where seasonal variation in food availability is typically a product of rainfall patterns and often not well-defined. Second, data on the relationship between food quality and physiological requirements of the animal are not always available. Finally, a range of other factors are also significant contributors to microbiome variation, most notably social structure, geographic site, and individuality (Probst *et al*. 2013). Thus, individuals need to be followed over time, and it is challenging to relate findings across primate populations from study sites where foods and climate vary dramatically. Indeed, a recent study found that host phylogenetic relationships were a better predictor of microbiome than was host dietary niche (e.g. folivory vs. frugivory) or geographic location. This association was attributed to anatomical and physiological differences that are phylogenetically conserved (Amato *et al*. 2019a).

We found that Taihangshan macaques have a relatively simple diet, comprising 57 different food items spread across 8 categories (Cui *et al*. 2019), compared with 69 food items in a single baboon observed over 30 days (Johnson *et al*. 2013) and 445 food items in the annual diet of a population of blue monkeys in Kenya (Takahashi *et al*. 2019). At our study site, availability of these foods changes according to strongly predictable seasonal factors and at each season macaques choose preferred food items (Cui *et al*. 2019). We observed marked distinctions in diet compositions for all 4 seasons. Food item analysis shows this can be hierarchically described with 2 distinct groups based on either acorn seeds (autumn/winter) or leaves (spring/summer) that are further separated into subgroups by differential use of herbs or woody plant parts giving 4 distinct clusters corresponding to the seasons (Fig. 1b). The microbiome analyses also showed 4 distinct clusters by season, but the effect of season on microbiome dynamics was more pronounced than in most other wild primate studies, especially with respect to summer. This could reflect either more strongly demarcated diet transitions in our study, or that all samples came from a single social group which may reduce inter-individual differences.

Although the microbiome pattern also showed 4 clusters corresponding to the seasons, the environmental drivers of these differences were captured differently by food item categories or nutrients. In dbRDA using nutrients, the overt separation of summer was strongly explained by AP and autumn was resolved from winter by fat intake. When dbRDA using food items as the fitted variable was performed, it did not identify leaf consumption as unifying driver of summer and spring communities, but did identify seeds as explanatory factor for autumn/winter. Collectively, this suggests the distinction of summer (biggest effect) is best explained by macronutrient intake patterns but that the distinction between autumn, winter, and spring is more usefully explained by food items. However, a question remains as to why nutrient intake poorly explained separation of spring samples and the autumn/winter samples. The simplest way to reconcile this is if a threshold level of protein energy intake is the major driver of microbiome response. We have previously shown in a mouse model with purified diet components that total protein and carbohydrate intake is an important driver of microbiome structure (Holmes *et al*. 2017). If true here with a natural diet, this model would imply that the threshold for a change in the microbiome structure has a very tight "tipping point" between the nutrient distributions of NP:AP of 3 and 2 that distinguishes the summer-type microbiome from the autumn/winter/spring ones. This would offer an explanation for the aberrant relationships of the samples from M3, M4, F1, and F2 that were collected at the boundaries of the 2016 and 2017 summer seasons.

It is useful to consider different ways to conceptualize dietary niches together with other recent observations on drivers of primate microbiomes. Amato *et al*. (2019a) found that across 18 species of wild nonhuman primates, representing both folivorous and non-folivorous dietary niches, host phylogeny was the strongest predictor of microbiome composition (Amato *et al*. 2019a). Dietary niche had a surprisingly minor effect, leading those authors to conclude that the influence of host physiology and anatomy (both correlated with phylogeny) have a substantially greater impact on primate gut microbiota than does dietary niche. This interpretation arises if the dietary niche is defined by food item type. Our data also show that food items were poor predictors (the 2 seasons with leaf-dominated diets did not share similar microbiome structure), but further revealed that patterns were seen if the dietary niche was described at the level of energy intake as macronutrients.

A benefit of our study is that it was performed in a non-invasive natural context, and we can be confident the results are not an artefact of laboratory manipulations (Aziz 2017). The trade-off, however, is that in observational studies, particularly in natural settings, some aspects of the methods inevitably involve assumptions. We used the conventional factor of 6.25 to estimate protein from nitrogen content, based on 16% nitrogen in amino acids and

derived from analyses of domesticated agricultural plants. A study of wild tropical plant parts found that the nitrogen content of the amino acids was closer to 19% (Milton & Dintzis 1981), but it remains to be determined if the same is true for temperate wild plants, or indeed whether different conversion factors apply for different species. Additionally, wild plants contain more non-protein nitrogen than domesticated agricultural plants, and this too might have introduced inaccuracy into our estimates of dietary protein. In estimating TNC by difference, we also might over-estimate carbohydrate by not considering organic compounds such as tannins; on the other hand, we might have underestimated carbohydrate energy because some carbohydrates such as pectins have energy value below the conversion factor we used of 16.7 kJ/g, and some oligosaccharides in plants are undigestible and so yield no energy to the animal. Whether and to what extent these commonly used methods can substantively affect the interpretation of field studies is an important question. Encouragingly, several studies that have made similar assumptions have recovered strong signals of macronutrient regulation in wild primates (e.g. Felton *et al*. 2009; Rothman *et al*. 2012; Johnson *et al*. 2013; Cui *et al*. 2018). This suggests that such approaches are an adequate proxy for nutrient compositions of wild foods to assess primate nutritional ecology.

Our results emphasize the importance of understanding nutrition in a multi-dimensional context, in which foods and diets are considered as mixtures, rather than single components (Raubenheimer *et al*. 2009), and the gut microbiome is considered part of the animal system. They further show that application of multi-dimensional approaches better explains ecological patterns than do the dominant foods in the diet. This re-affirms the importance of adopting a multi-level definition of the dietary niche (Machovsky-Capuska *et al*. 2016), in which nutrient compositions of foods, identities of available foods, and foraging strategies to integrate those foods into diets play nominally different, but interacting roles, in defining (and observing) ecological niches. As mentioned in the introduction, giant pandas are an example of how this can reconcile animal and microbiome traits in nutritional ecology. In terms of their food items and foraging behavior, giant pandas are exclusively herbivorous, yet they are phylogenetically related to carnivores. Nie *et al*. (2015) showed that giant pandas ingest a diet which clusters in a macronutrient space with the diets of hypercarnivores and is markedly distinct from herbivores (Nie *et al*. 2015). This might explain why those enigmatic animals, even though extreme specialist herbivores, have retained some carnivore-associated traits, including their gut anatomy and a microbiome that is remarkably low in fiber-degraders (Xue *et al*. 2015).

Similarly, the multi-dimensional approach provides new lenses for understanding the dietary generalist–specialist spectrum (Machovsky-Capuska *et al*. 2016). The relatively low number of foods eaten by macaques in our study population (discussed above) places them closer to the specialist end of the primate dietary spectrum. However, previous work in this same population showed that, despite the relatively low number of foods in their diet, these monkeys show wide tolerance for inter-annual variation in the macronutritional composition of the diet, predicted by ecological theory to be a characteristic of generalist species (Cui *et al*. 2018, 2019). Conversely, Takahashi *et al*. (2019) showed that tropical blue monkeys (*Cercopithecus mitis*) eat a large number of different foods (445), and yet maintain daily macronutrient intakes within tight limits. These observations suggest that the classification of animals on the generalist-specialist spectrum might differ at the levels of number of foods eaten and the range of dietary macronutritional compositions tolerated, as predicted on theoretical grounds (Raubenheimer & Simpson 1999).

Relatedly, the multidimensional approach might also help to reconcile the spectrum of ecological niches within species complexes. As a group, macaques are capable of subsisting on a considerable range of different foods. After humans, the rhesus macaque species complex is the most geographically widespread and ecologically generalist of all primates (Fooden 2000; Cui *et al*. 2018). This suggests that the generalist pattern of macronutrient handling observed in our study population might be a species-level characteristic that has enabled individual populations to inhabit disparate food environments some of which, such as that of the Taihangshan macaques, are characterized by low food diversity. This is interesting because it highlights an important nuance in niche theory, distinguishing between individual-, population-, and species-level adaptations to fluctuating resource availability (Holt 2009).

Humans also show geographically separated populations that historically had distinct differences in available foods. As modernization has changed the human nutrient environment, an epidemic of nutrition-related chronic disease has emerged and changes in the microbiome are strongly associated with this. Significantly, susceptibility to these diseases shows differences between populations of different ethno-geographic backgrounds (Zimmet 2017). Thus, improved understanding of the drivers and

consequences of variation in the gut microbiomes of animals is a high priority for both ecology and human public health.

Recently an ecological approach to diabetes management has been proposed in which it was noted that the concept of ecological guilds, sets of bacteria that responded similarly to diet, was a more useful way to identify correlations between diet, microbiome, and clinical outcomes (Zhao *et al*. 2018). Our data here also show gut microbiome responses in wild animal populations can be simplified at the guild level. An important challenge for future research is to improve our understanding of whether and how gut microbiome responses to animal diets in the wild complement other regulatory strategies of the animal. We expect that new insights would arise from study of how gut microbial responses integrate with behavioral and physiological strategies tolerating macronutritional imbalance in rhesus macaque populations across ecologically diverse habitats.

ACKNOWLEDGMENTS

We thank Jiyuan Administration of Taihangshan Macaque National Nature Reserve for permission to conduct field work and logistic support throughout this study. We also thank Prof. Li Baoguo, Prof. Guo Songtao, and Dr. Hou Rong (Northwest University, China) for their assistance with the nutritional analyses. This work was supported by the National Natural Science Foundation of China (No. 30970378, 31170503), China Postdoctoral Sciences Foundation (No. 2019M662520), and the State Scholarship Fund from China Scholarship Council (No. 201707040024).

CONFLICT OF INTEREST

The authors confirm no conflict of interest.

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 A) Average daily nutrient intake for animals at all 4 seasons.

Figure S2 Proportional occurrence of selected taxa across dataset.

Table S1 A total 48 fecal samples were obtained from 12 identified adult individuals (6 males and 6 females) from 4 seasons.

Table S2 Daily nutrient or food item intakes of wild monkeys

Cite this article as:

Cui Z, Holmes AJ, Zhang W *et al*. (2022). Seasonal diet and microbiome shifts in wild rhesus macaques are better correlated at the level of nutrient components than food items. *American Journal of Primatology* **17**, 1147–61. https://doi.org/10.1111/1749-4877.12601