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RESEARCH ARTICLE

Gut microbiota composition of Japanese macaques associates with extent of human encroachment

Wanyi Lee[1] | Takashi Hayakawa[2,3] | Mieko Kiyono[4] | Naoto Yamabata[5] | Goro Hanya[1]

[1]Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan

[2]Faculty of Environmental Earth Science, Hokkaido University, Sapporo, Hokkaido, Japan

[3]Japan Monkey Centre, Inuyama, Aichi, Japan

[4]Graduate School of Human Development and Environment, Kobe University, Kobe, Hyogo, Japan

[5]Institute of Natural and Environmental Sciences, University of Hyogo, Sanda, Hyogo, Japan

Correspondence
Wanyi Lee, Department of Ecology and Social Evolution, Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan.
Email: wanyi23333@gmail.com

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Abstract

In recent decades, human–wildlife interaction and associated anthropogenic food provisioning has been increasing and becoming more severe due to fast population growth and urban development. Noting the role of the gut microbiome in host physiology like nutrition and health, it is thus essential to understand how human–wildlife interactions and availability of anthropogenic food in habitats can affect an animal's gut microbiome. This study, therefore, set out to examine the gut microbiota of Japanese macaques (*Macaca fuscata*) with varying accessibility to anthropogenic food and the possibility of using gut microbiota as indicator for macaques' reliance on anthropogenic food. Using 16S ribosomal RNA gene sequencing, we described the microbial composition of Japanese macaques experiencing different types of human disturbance and anthropogenic food availability—captive, provisioned, crop-raiding, and wild. In terms of alpha diversity, our results showed that observed richness of gut microbiota did not differ significantly between disturbance types but among collection sites, whereas Shannon diversity index differed by both disturbance types and sites. In terms of beta diversity, captive populations harbored the most distinctive gut microbial composition, and had the greatest difference compared with wild populations. Whereas for provisioned and crop-raiding groups, the macaques exhibited intermediate microbiota between wild and captive. We identified several potential bacterial taxa at different taxonomic ranks whose abundance potentially could help in assessing macaques' accessibility to anthropogenic food. This study revealed the flexibility of the gut microbiome of Japanese macaques and provided possible indices based on the gut microbiome profile in assessing macaques' accessibility to/reliance on anthropogenic foods.

KEYWORDS
gut microbiome, human disturbance, *Macaca*, primate

1 | INTRODUCTION

The gut microbiome is the community of microorganisms residing in the gastrointestinal tract and is actively involved in many aspects of host physiology such as energy harvest, nutrition (Amato et al., 2014, 2015a), behavior (Davari, Talaei, & Alaei, 2013), and immune system response (Round & Mazmanian, 2009). While the gut microbiome strongly influences hosts' digestive efficiency and health, host diet in turn affects the gut microbiome (Clayton et al., 2018a; 2018b; Ley et al., 2008a; Muegge et al., 2011). Host diet affects the metabolic activities of the gut microbes by providing different substrates and nutrition, thus influencing the composition and functions of the gut microbiome. Many studies

based on feeding experiments have revealed that gut microbiota is related to the types and the macronutrient profile of food, as exemplified by the distinct human and non-human primate (NHP) gut microbial communities in response to Western and non-Western diets (Amato et al., 2015b; Wu et al., 2011). In particular, humans who consume Western diets which is low in fiber, high in protein and fat exhibited increased *Bacteroides*, whereas those who consumed non-Western diets had increased abundance of *Prevotella*. Compared with feeding based experiments of humans and lab animals, wild animals exhibit even wider dietary variation. Food sources of wild NHPs vary temporally and spatially, in relation to the local climate, habitat type, plant phenology, and so on. Corresponding to temporal and spatial dietary difference, composition and function of NHP gut microbiome were found to vary across seasons and habitats (Amato et al., 2015a; Barelli et al., 2015; Bennett et al., 2016; Hicks et al., 2018; Mallott, Amato, Garber, & Malhi, 2018; Sun et al., 2016; Zhao et al., 2018). A notable example of diet–gut microbiome relationship across seasons was revealed by Amato et al. (2015a). This study showed that gut microbiome shifts in composition across seasons and functions to compensate for seasonal reduction in howler energy intake. Because the host–gut microbiome relationship has evolved in the natural environments, studies on wild, free-ranging animals will allow more thorough understanding of the role of environmental factors in this relationship.

On the global scale, human disturbance like agriculture and tourism have been increasingly affecting ecology and behavior of NHPs (Fuentes, 2006; Fuentes & Hockings, 2010). In particular, such human disturbance made anthropogenic food available to NHP via directly provisioning or crop-raiding thus could easily influence foraging behavior and nutritional intake of the NHPs (Hill, 2017; Ilham, Nurdin, & Tsuji, 2017; McKinney, 2011; Sha & Hanya, 2013). In some cases, anthropogenic food could constitute as much as 70% of NHP's total diet (Ilham et al., 2017). Whereas for captive individuals, the diet is managed by the keepers and is predominantly composed of commercial monkey chow for ease of management (Dierenfeld, 1997; Jaman & Huffman, 2008). From a nutritional aspect, monkey chow and the food enhancement from cropland and tourism tend to have lower fiber and higher digestible carbohydrates and energy value than wild foods (Clayton et al., 2018a; 2018b; Riley, Tolbert, & Farida, 2013). Such dietary shift likely induces significant changes in gut microbiome composition. Indeed, previous studies have revealed a general pattern of NHP gut microbiome composition becomes altered with decreased dietary diversity in captive environments (Clayton et al., 2016; 2018a; Hayakawa, Nathan, et al., 2018; McKenzie et al., 2017). Likewise, the gut microbiome of captive NHPs is less diverse and shows signs of humanization, converging toward the modern human microbiome (Clayton et al., 2016). However, captivity is not the only human activities influencing NHPs. To further understand how the gut microbiome of wild animals could be affected by anthropogenic activities, we examined the gut microbiome of NHPs under several human-disturbed habitats with varying availability of anthropogenic food.

In this sense, the *Macaca* genus serves as a suitable study subject due to their extensive distribution and close proximity to humans (Priston & McLennan, 2013). In the present study, we focused on Japanese macaques (*Macaca fuscata*), an endemic primate species widely distributed in Japanese archipelago. Human–monkey interactions come in varying forms in Japan, including but not limited to conditions of captivity, provisioning, and crop-raiding (Hill & Webber, 2010; Nakagawa, Nakamichi, & Sugiura, 2010; Yamagiwa & Hill, 1998). Human-disturbed Japanese macaques may similarly influence the gut microbiome with the associated environmental and dietary shifts. In the wild, Japanese macaques mainly feed on plant parts like leaves, flowers, fruits, buds, and bark but the proportion of each food item differs by seasons and regions (Tsuji, 2010). For example, fruits are the primary food for macaques inhabiting the Yakushima lowland (Hill, 1997), whereas for macaques in the Yakushima highland, leaves are the most consumed food (Hanya, 2004a). On the contrary, captive, provisioned, and crop-raiding macaques feed on anthropogenic foods, for example, commercial monkey chows and crops with varying proportion among populations. At the extreme, captive macaques are completely dependent on anthropogenic food because they are limited by the enclosures or cages. Although researchers have noted the effect of human disturbance and anthropogenic food availability on NHPs' behavior, previous studies rarely examine varying degrees of human disturbance on single species, Japanese macaques hence are suitable study subjects since they are commensal with humans in many of their habitats.

Here, we aim to understand how disturbance and associated anthropogenic food enhancement may affect the gut microbiome profile of Japanese macaques. Specifically, we described and compared the gut microbiota of macaques with different accessibility to anthropogenic food under different human disturbance types, that is, wild, provisioned, crop-raiding, and captive. With this data set, we also examined the bacterial taxa whose relative abundance is associated with the anthropogenic food availability in habitats. With reference to previous studies (Amato et al., 2013, 2015b; Clayton et al., 2016, 2018a; McKenzie et al., 2017), we contrasted the patterns observed in Japanese macaques with other primate species. As an outcome of their diverse, fiber-rich diet, we hypothesized that the gut microbiome of wild macaques would be more diverse and enriched in microbes specialized for fiber digestion. Whereas for the anthropogenic food-enhanced macaques, that is, captive, crop-raiding, and provisioned macaques, their gut microbiota would be less diverse and distinctive from that of the wild macaques based on the availability of anthropogenic food in habitats.

2 | METHODS

2.1 | Collection of fecal samples from Japanese macaques

Fecal samples were collected from Japanese macaques. Based on human disturbance types or diet the populations experienced, the monkeys were categorized as wild, provisioned, crop-raiding, or captive (Tables 1 and S1). Samples from wild macaques were collected from free-ranging groups in highland and lowland areas of

TABLE 1 Basic information of sample collection sites

Disturbance types	Site	Diet	Conspecific contact	Living environment	Close interaction with humans
Captive	PRI cage	Simple diet; monkey chow	×	Artificial	High
Captive	PRI enclosure	Simple diet; monkey chow	◯	Artificial	High
Provisioned	Shodoshima	Intensive provisioning (3–4/day)	◯	Partly artificial	Medium
Crop-raiding	Suzuka-shi, Mie prefecture	No record; natural and agricultural food source	◯	Generally wild	Medium
Provisioned	Koshima	Controlled provisioning (2/week)	◯	Wild	Medium
Wild	Yakushima lowland	Frugivory based	◯	Wild	Rare
Wild	Yakushima highland	Folivory based	◯	Wild	Rare

Abbreviation: PRI, Primate Research Institute, Kyoto University.
◯ and X means yes and no.

Yakushima Island, Kagoshima Prefecture, Japan (30°N, 131°E) in August 2013 and May 2017, respectively. Samples from provisioned macaques were collected from free-ranging monkeys in Koshima Islet, Miyazaki prefecture (31°22′N, 131°26′E) in April 2017 and Shodoshima, Kagawa prefecture, Japan in May 2017. Samples from crop-raiding macaques were collected from free-ranging groups in Suzuka, Mie prefecture (N34° 55′ E136° 28′) in July 2017. Japanese macaques in Shodoshima are intensively provisioned three to four times a day to make visible to visitors (Leca, Gunst, & Huffman, 2008). On the other hand, provisioning in Koshima is relatively limited in frequency and quantity, which occurs about two times a week, and the macaques spent similar amount of time foraging for natural foods (Go, 2010; Leca et al., 2008). Samples from captive macaques were collected from individuals living in individual cages and individuals living as a group in enclosures at Primate Research Institute, Kyoto University (PRI). The diet of captive individuals is composed of monkey chow and minor food items like sweet potatoes (Jaman & Huffman, 2008; Jaman, Huffman, & Takemoto, 2010). For each site, 10 fecal samples were randomly collected. During sampling, we also collected fecal samples from unknown individuals with unknown age-sex class, since our goal is to predict macaques' reliance on anthropogenic food irrespective of age and sex.

2.2 | Sample storage, DNA purification, 16S ribosomal RNA (rRNA) amplification and sequencing

Our method followed Hayakawa, Nathan, et al. (2018) with slight modification. All fecal samples ($N = 70$) were collected immediately after defecation using sterilized cotton swab, then stored in 1-ml lysis buffer (0.5% sodium dodecyl sulfate, 100 mM ethylenediaminetetraacetic acid (pH 8.0), 100 mM Tris-HCl (pH 8.0), and 10 mM NaCl), where the lysis buffer provided an appropriate storage medium for bacterial DNA as well as easy to handle and cost-effective. After bead-beating and centrifuged at 20,000*g* for 1 min, each fecal sample was mixed with 1,000 μl InhibitEX buffer of the QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany). The mixture was centrifuged at 20,000*g* for 1 min, 600 μl of the supernatant was mixed with 25 μl proteinase K and 600 μl Buffer AL (Qiagen GmbH, Hilden, Germany) and followed by the manufacture's protocols to purify the fecal DNA. DNA concentration was estimated with Qubit dsDNA HS Assay Kit and a Qubit fluorometer (Thermo Fisher Scientific). We amplified the V3-V4 region of 16S rRNA gene with primers as follows: S-D-Bact-0341-b-S-17 (forward) 5′-CCTACGGGNGGCWGCAG-3′ and S-D-Bact-0785-a-A-21 (reverse) 5′-GACTACHVGGGTATCTAATCC-3′ (Klindworth et al., 2013). Polymerase chain reaction (PCR) products were purified using Agencourt AMPure XP beads (Beckman Coulter, Inc., Carlsbad, CA). Using the Illumina Nextera XT Index Kit (Illumina, Inc., San Diego, CA), specific dual indices and sequencing adapters were attached to each amplicon by PCR. Products were mixed in the same amount of DNA concentrations to form the pooled sequencing library. Fragment size distribution of the library was estimated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., La Jolla, CA). The library was diluted to 15 pM and subjected to a sequencing run and 30% PhiX spike-in on an Illumina MiSeq sequencing platform using the MiSeq Reagent Kit v3 (600 cycles) (Illumina, Inc., San Diego, CA). The read lengths from the MiSeq run were 301 bp (forward sequences), 8 bp (forward indices), 8 bp (reverse indices), and 301 bp (reverse sequences). The data have been deposited in the DNA Data Bank of Japan database with accession number DRP005397. This study was approved by PRI with permission number 2017-161-07 and conducted in accordance to Primate Research Institute's Guideline for Animal Health and Welfare. Our research is complied with the American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates.

2.3 | Data analysis

Raw sequences were processed following steps described in Hayakawa, Nathan, et al. (2018) using software Claident v0.2.2016.4.7 (Tanabe & Toju, 2013) and QIIME2 (Caporaso et al., 2010). Demultiplexed sequences with quality score <30 were discarded, then merged using PEAR v0.9.3 (http://sco.h-its.org/exelixis/web/software/pear/) with setting p .0001 and u 0). To pick operational taxonomic units (OTUs), read sequences were clustered at 97% cutoff similarity level. For taxonomic identification, OTUs were assigned through the

ribosomal database project classifier at 50% confidence threshold with GreenGenes v13_8 as the reference database. The sequencing read set of each sample was rarefied to the minimum read number among the analyzed samples (13,100). Rarefaction curves were plotted and slope of rarefied curves for each sample were checked using "rarecurve" and "rareslope" function of R package *vegan* (Oksanen et al., 2013). Statistical analyses were performed in R Version 3.4.3 (R Core Team, 2018). Rarefied data set was analyzed without pruning any bacterial taxa. Alpha and beta diversity were calculated using R package *phyloseq* (McMurdie & Holmes, 2013). R package *dunn.test* was used to analyze the differences of alpha diversity indexes among groups. To construct a phylogenetic tree of the OTUs, we used the built-in function align-to-tree-mafft-fasttree of QIIME. For multivariate analysis of microbiome composition, we constructed nonmetric multi-dimensional scaling (NMDS) by Bray-Curtis and principal coordinate analysis (PCoA) plots by weighted and unweighted UniFrac indices through *phyoseq* in R. To find the indicator bacterial taxa for the level of human disturbance experienced by the macaques, we analyzed the data set at different taxonomic rank using regression random forest model through R packages *randomForest* and *caret*. We use single number to mark the samples and check if the picked bacterial taxa could correctly predict the disturbance level of the samples. Specifically, the levels are captive-cage and enclosure (a), intensively provisioned-Shodoshima (b), crop-raiding-Suzuka (c), less provisioned-Koshima (d), wild-Yakushima lowland (e), wild-Yakushima highland (f). To confirm the reliability of picked bacterial taxa in identifying disturbance level experienced by the macaques, we also employed leave-one-out validation.

3 | RESULTS

3.1 | General characteristics of gut microbiome of Japanese macaques

After removing samples with rarefaction curve slope <0.01 (one Yakushima highland, one Yakushima lowland, one cage, one Koshima), we detected totally 2,125 OTUs at 97% sequence similarity in the remaining 66 samples (rarefaction curve slope: 0.0021–0.0085; Figure S1). OTUs identified were from 35 phyla, 74 classes, 109 orders, and 165 families. Average observed OTU richness was 362 ± 64/sample, ranging from 193 to 461 OTUs (Figure 2a and Table S1). The average unclassified rates of OTUs were 0.08% at the phylum level and 32.6% at the genus level. At the phylum level, Firmicutes and Bacteroidetes the gut microbiome of Japanese macaques by 59.95% and 29.50% (Figure 1 and Table 2). The other dominant phyla were Proteobacteria (4.53%) and Spirochetes (2.19%) and Verrucomicrobia (0.98%). At the genus level, *Prevotella* accounts for 20.70%, followed by *Faecalibacterium* (7.98%) and *Oscillospira* (7.31%).

3.2 | Variation of gut microbiota among different human disturbance types: Alpha diversity

Overall, indices for alpha diversity, observed richness and Shannon diversity index, showed different patterns. OTU richness did not differ by disturbance types (Kruskal-Wallis $\chi^2 = 0.7619$, $df = 3$, $p = .86$; Figure 2a). Compared to that, Shannon diversity index differs by

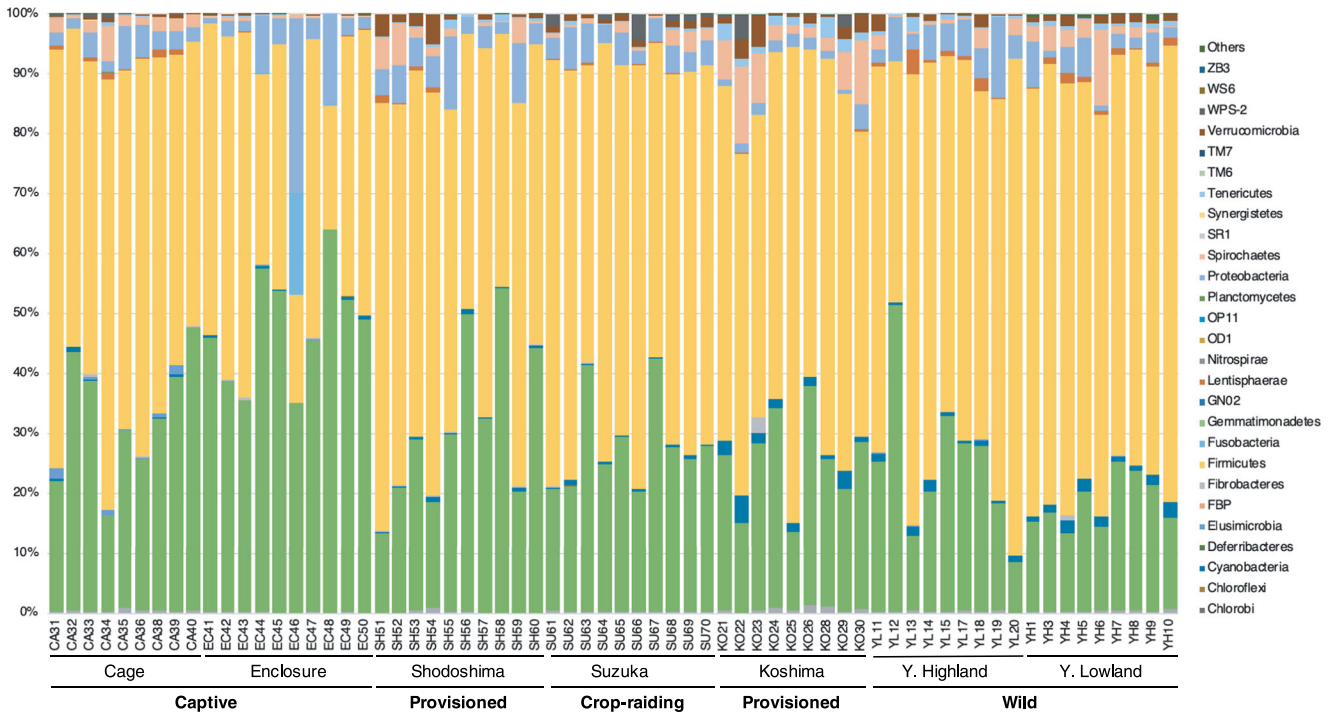

FIGURE 1 Relative abundance of gut bacterial taxa at phylum level. Abbreviation represents the collection sites. CA, Cage; EN, Enclosure; KO, Koshima; SH, Shodoshima; SU, Suzuka; YL, Yakushima lowland; YH, Yakushima highland

TABLE 2 Relative abundance of dominant gut microbial phyla in Japanese macaques experiencing different human disturbances (order from most abundant to least abundant)

Phylum	Captive (%)	Crop-raiding (%)	Provisioned (%)	Wild (%)	Average (%)
Firmicutes	50.25	63.30	58.95	67.31	59.95
Bacteroidetes	40.46	27.91	28.15	21.46	29.50
Proteobacteria	5.39	4.35	3.79	4.61	4.53
Spirochetes	1.31	1.28	3.94	2.23	2.19
Verrucomicrobia	0.23	1.05	1.68	0.96	0.98
Cyanobacteria	0.23	0.38	1.14	1.27	0.76
Tenericutes	0.13	0.22	1.09	0.65	0.52
Lentisphaerae	0.29	0.18	0.20	0.89	0.39
WPS-2	0.07	0.95	0.32	0.00	0.34
Actinobacteria	0.29	0.26	0.43	0.35	0.33
Fusobacteria	0.92	0.00	0.00	0.00	0.23
Fibrobacteres	0.10	0.04	0.21	0.06	0.10
Elusimicrobia	0.30	0.00	0.00	0.05	0.09
Unassigned	0.01	0.05	0.10	0.14	0.08

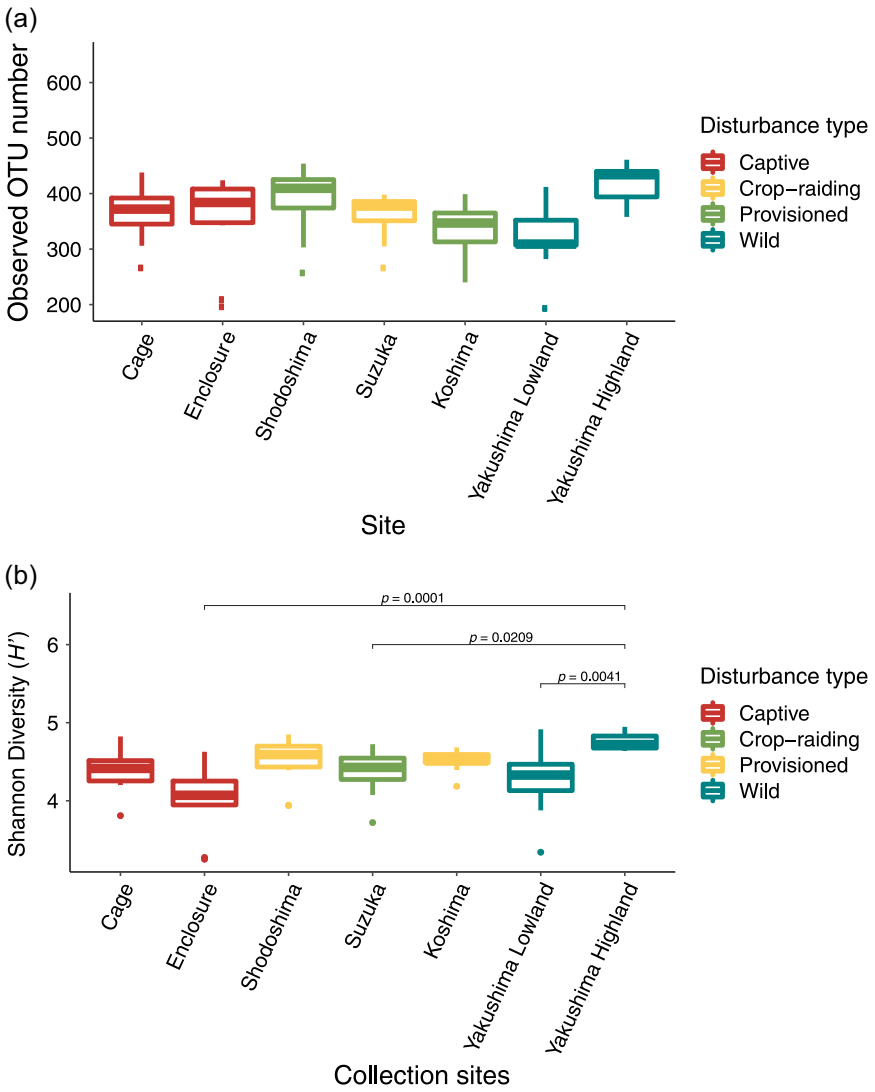

FIGURE 2 (a) Observed OTU richness of Japanese macaques. Color indicates human disturbance type. (b) Shannon diversity index of Japanese macaques. Color indicates human disturbance type. OTU, operational taxonomic unit

disturbance types (Kruskal-Wallis $\chi^2 = 8.5960$, $df = 3$, $p = .04$; Figure 2b). Between different disturbance types, Shannon diversity index of captive macaques' gut microbiome was significantly lower than that of the wild macaques (Dunn's rank sum test corrected by Bonferroni, captive vs. wild, $p = .0275$). Except for that, no other pairwise comparisons showed any significant difference.

3.3 | Variation of gut microbiota among different human disturbance types: Beta diversity

According to multivariate analysis based on NMDS plot by Bray-Curtis and PCoA plots by unweighted UniFrac, individuals from the same collection sites always possessed more similar microbial communities (Figure 3). We performed permutational multivariate analysis of variance (PERMANOVA) tests to assess the degree of variation explained by disturbance type and collection site. Site where samples were collected was a good predictor for gut microbial community (PERMANOVA, Bray-Curtis, $R^2 = 0.4926$, $p < .001$; unweighted UniFrac, $R^2 = 0.4529$, $p < .001$), whereas disturbance type explained less of the variation (PERMANOVA, Bray-Curtis, $R^2 = 0.3576$, $p < .001$; unweighted UniFrac, $R^2 = 0.3284$, $p < .001$). In NMDS plot and PCoA plot based on unweighted UniFrac, samples with different human disturbance level/accessibility to anthropogenic food were separated on the first dimension, in the order PRI cage = PRI enclosure > Shodoshima > Suzuka > Koshima > Yakushima lowland > Yakushima highland. Samples from individuals living in cages and enclosures clustered together and were distinct from the other individuals. On the contrary, samples from the Yakushima lowland and highland were situated at the farthest end, away from the captive cluster. Provisioned and crop-raiding individuals occupied

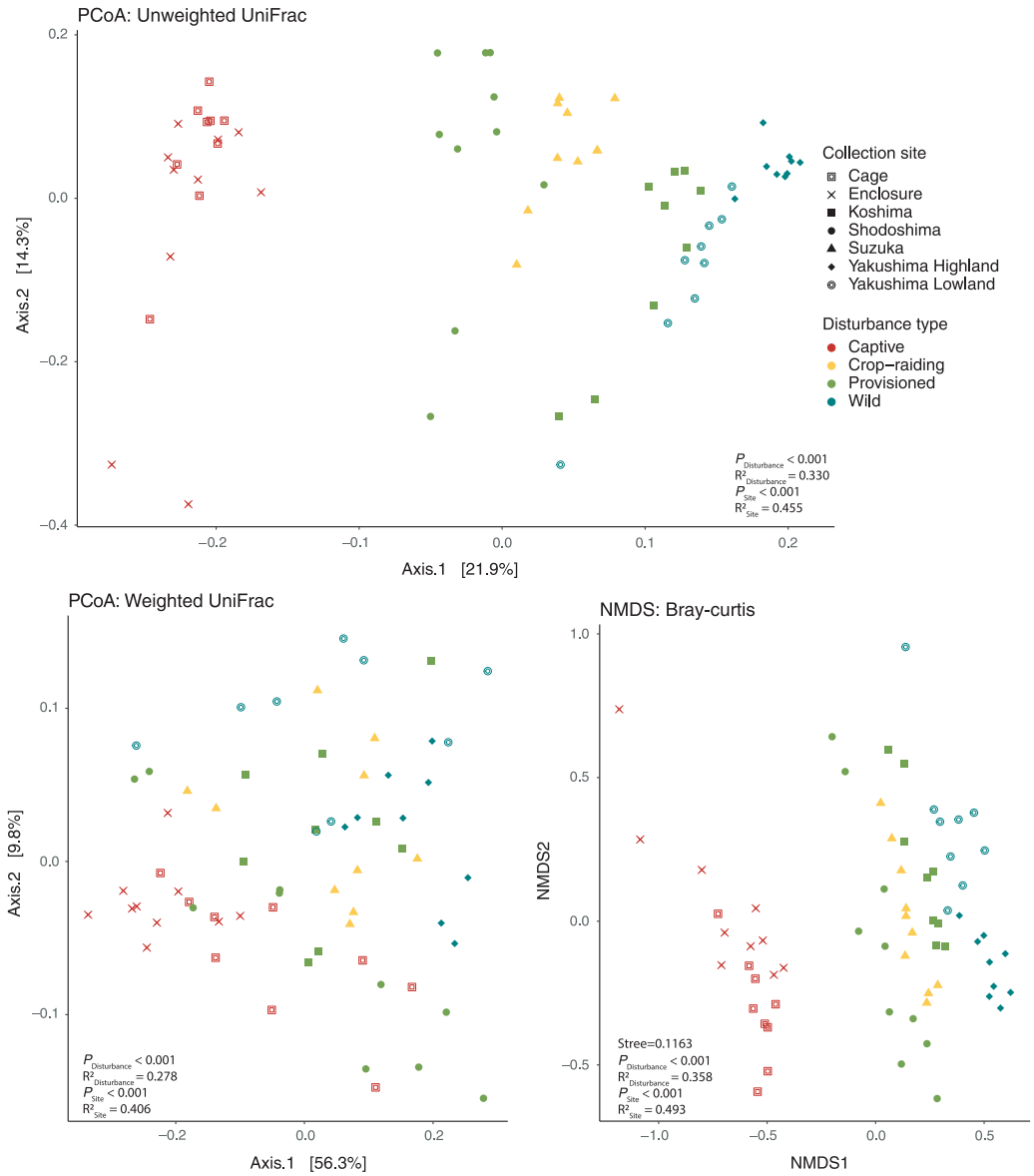

FIGURE 3 NMDS and PCoA plots based UniFrac distance for macaques' gut bacterial communities. NMDS, nonmetric multidimensional scaling; PCoA, principal coordinate analysis

the intermediate position of captive and wild. On the second dimension of NMDS plot, samples with similar accessibility to anthropogenic food but from different sites were distinguishable by second dimension of NMDS2 but not by PC2. Yakushima highland samples were separated from Yakushima lowland, and samples from PRI cages were separated from those collected in PRI enclosures. The difference between the NMDS plot and PCoA plot by unweighted UniFrac may be related to phylogenetic closeness of OTUs shared by cage and enclosure samples that could not be detected through NMDS plot. In PCoA plot based on weighted UniFrac, only captive samples were weakly clustered, despite the significant difference by sites and disturbance type revealed by PERMANOVA (weighted UniFrac, site, $R^2 = 0.4187$, $p < .001$; disturbance, $R^2 = 0.2872$, $p < .001$; Figure 3).

3.4 | Gut bacterial taxa related to availability of anthropogenic food to macaques

To find the potential bacterial indicator, we analyzed using random forest model and check if the picked bacterial taxa could correctly predict the disturbance level of the samples. Specifically, the levels are captive-cage and enclosure, intensively provisioned-Shodoshima, crop-raiding-Suzuka, less provisioned-Koshima, wild-Yakushima lowland, and wild-Yakushima highland (Figure 3). Overall, the accuracy of indicator taxa from lower taxonomic ranks is higher than those from higher taxonomic ranks. Models using families (OOB rate = 3.03%, accuracy = 1.00, $\kappa = 1.00$) predicts the food reliance level better than orders (OOB rate = 10.61%, Accuracy = 1.00, $\kappa = 1.00$), classes (OBB rate = 15.15%, Accuracy = 1.00, $\kappa = 1.00$), and phyla (OBB rate = 22.73%, Accuracy = 1.00, $\kappa = 1.00$). To identify macaques' accessibility to anthropogenic food, information at lower taxonomic rank may serve good indicator, since they may provide more diet-specific features. Whereas as when we use information from higher taxonomic ranks, some characteristics of the gut bacteria may be overlooked. In our data set, were identified class Bacilli, class Chloroplast, order Lactobacillales, and family Bacteroidaceae to be the most important indicator taxa, with mean decrease in accuracy higher than 0.04 (Table S2 and Figure 4).

Several indicators used in previous studies on gut microbiome, that is, Firmicutes to Bacteroidetes ratio, Chloroplast, *Bacteroides*, and *Prevotella* showed varying response to human disturbance and anthropogenic food enhancement. For the Firmicutes to Bacteroidetes ratio, macaques from wild populations had the highest value, followed by provisioned and crop-raiding, with the lowest in captive populations (Kruskal-Wallis rank sum test $\chi^2 = 21.2245$, $df = 3$, $p < .0001$; Dunn's rank sum test corrected by Bonferroni, Captive vs. Crop-raiding, $p = .0514$; Captive vs. Provisioned, $p = .0362$; Captive vs. Wild, $p < .0001$; Crop-raiding vs. Provisioned, $p = 1.000$; Crop-raiding vs. Wild, $p = .4412$; Provisioned vs. Wild, $p = .1096$; Figure 1). We also examined reads classified as Chloroplast, as it is used as an indicator of host fiber intake (Clayton et al., 2016, 2018a). Despite the intriguingly high abundance in Koshima samples, abundance of Chloroplast is negatively related to availability of anthropogenic food for the macaques, with captive samples with significantly low Chloroplast abundance (Kruskal-Wallis rank sum

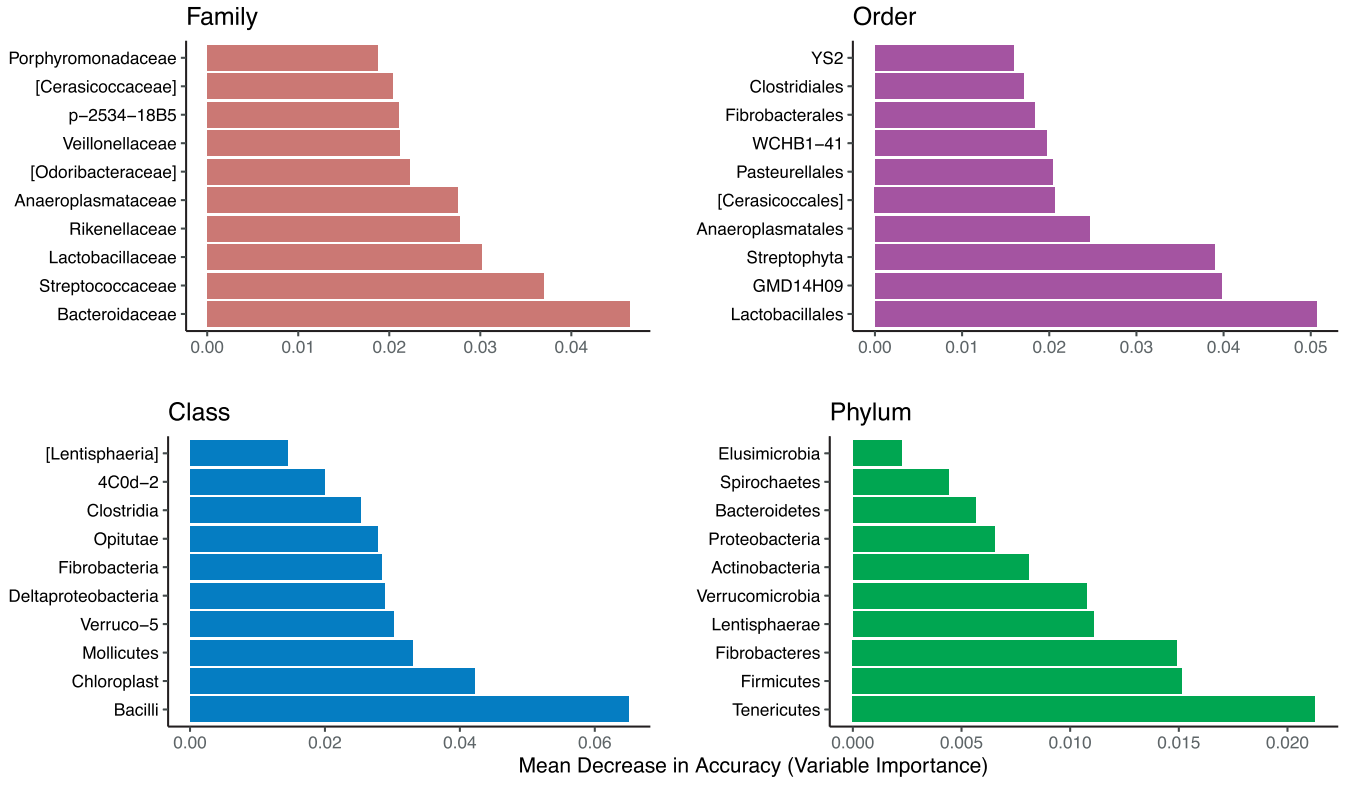

FIGURE 4 Top 10 bacterial taxa important in assessing macaques' reliance on anthropogenic food at each taxonomic rank (phylum, class, order, and family)

test $\chi^2 = 34.2052$, $df = 3$, $p < .0001$; Dunn's rank sum test corrected by Bonferroni, Captive vs. Crop-raiding, $p = .0048$; Captive vs. Provisioned, $p < .0001$; Captive vs. Wild, $p < .0001$; Crop-raiding vs. Provisioned, $p = .6495$; Crop-raiding vs. Wild, $p = 1.000$; Provisioned vs. Wild, $p = 1.000$; Figure 5). For *Prevotella*, one of the dominant human gut microbial genera, significantly higher abundance was found in captive individuals, while crop-raiding, provisioned, and wild individuals had similar abundance (Kruskal-Wallis $\chi^2 = 15.2951$, $df = 3$, $p < .001$; Dunn's rank sum test corrected by Bonferroni, Captive vs. Crop-raiding, $p = .0171$; Captive vs. Provisioned, $p = .0205$; Captive vs. Wild, $p = .0011$; Crop-raiding vs. Provisioned, $p = 1.000$; Crop-raiding vs. Wild, $p = 1.000$; Provisioned vs. Wild, $p = 1.000$; Figure 6). Whereas for *Bacteroides*, another dominant human gut microbial genus, wild harbored the highest abundance, followed by captive macaques, and the lowest abundance was found in crop-raiding and provisioned macaques (Kruskal-Wallis $\chi^2 = 15.295$, $df = 3$, $p = .002$; Dunn's rank sum test corrected by Bonferroni, Captive vs. Crop-raiding, $p = .0007$; Captive vs. Provisioned, $p = .0003$; Captive vs. Wild, $p = .2127$; Crop-raiding vs. Provisioned, $p = 1.000$; Crop-raiding vs. Wild, $p < .0001$; Provisioned vs. Wild, $p < .0001$; Figure 6). However, *Bacteroides* generally were not abundantly present in the gut of Japanese macaques; the provisioned and crop-raiding individuals had low or sometimes no presence of *Bacteroides* spp.

4 | DISCUSSION

4.1 | General characteristics of gut microbiome profile of Japanese macaques

At the phylum level, the gut microbiome of Japanese macaques was dominated by Firmicutes and Bacteroidetes. Microbes from phyla Proteobacteria and Spirochetes were detected with lower abundance. At the genus level, *Prevotella* was the most dominant. Our findings of gut microbiome composition were consistent with previous studies on Japanese macaque gut microbiome (Hayakawa, Nathan, et al., 2018; Ma et al., 2014). In previous studies, Firmicutes and Bacteroidetes were the most abundant phyla constituting approximately 90% of the Japanese macaques' gut. Likewise, they also found Spriochaetes and Proteobacteria at minor abundance. However, previous studies on Japanese macaques focused on captive samples mostly ($n = 2$ from Hayakawa, Sawada et al., 2018; $n = 97$ from Ma et al., 2014) while limited samples were from wild, free-ranging individuals ($n = 2$ from Hayakawa, Nathan, et al., 2018).

Overall, the gut microbiome profile of Japanese macaques is similar to other macaques but different from great apes and humans. Firmicutes and Bacteroidetes are the two most dominant phyla in the mammalian gut (Clayton et al., 2018a; Ley et al., 2008a; 2008b). In this sense, Japanese macaques and other primate species including humans are similar, since Firmicutes and Bacteroidetes make up a great proportion of the gut microbiome. For hosts from the *Macaca* genus, Firmicutes, Bacteroidetes, Proteobacteria, and Spirochetes are the four most abundant bacterial phyla detected; in addition to Japanese macaques, these bacterial phyla also constitute a major part of the gut microbiota for captive *Macaca mulatta* (McKenna et al., 2008; Yasuda et al., 2015), captive *Macaca fascicularis* (Li et al., 2018), and wild *Macaca thibetana* (Sun et al., 2016).

Compared with macaques, the four most common phyla in gut microbiota of great apes, that is, bonobo, chimpanzees, and gorillas are Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria (Moeller et al., 2013). Similarly in humans, these four bacterial phyla are the majority of gut microbiota but the relative abundance varies with dietary habits (Arumugam et al., 2011; Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; De Filippo et al., 2010). Human, non-human apes, and macaque gut microbiome is distinctive in the presence of phyla Spirochetes, which tends to be rare in human and non-human ape guts. Also, genus *Bacteroides* is considered a major component of the human gut microbiota, but only minor in the macaque gut. Instead, *Prevotella*, another dominant bacterial genus for the human gut, is the most dominant genus for macaques. This difference was also noted by McKenna et al. (2008), who compared the gut microbiome profile of rhesus macaques with that of humans.

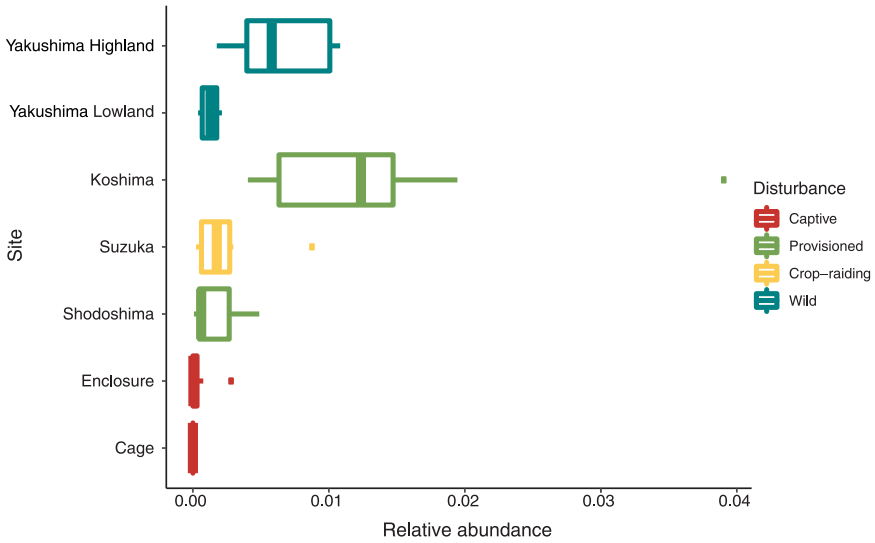

FIGURE 5 Relative abundance of Chloroplast

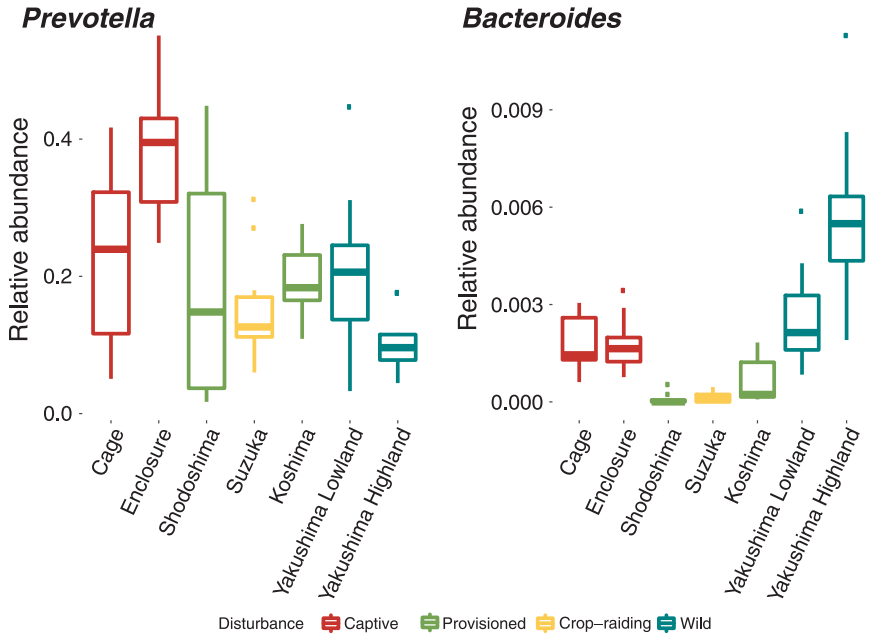

FIGURE 6 Relative abundance of the dominant bacterial genera in human, *Prevotella* and *Bacteroides*

4.2 | Effect of anthropogenic food availability on the gut microbiome of Japanese macaques: Similarity with other species

Our result showed that anthropogenic food availability in habitats of Japanese macaques and the associated dietary change was correlated with altered gut microbiota. A gradual change of gut microbiome composition was detected from macaques heavily relied on anthropogenic food (captive) to those relied on natural foods (wild). However, gut microbial diversity did not necessarily decrease along with increasing availability of anthropogenic food in disturbed conditions; Shannon diversity index differed between captive and wild populations, but observed richness was similar among disturbance types.

Within the three different types of human disturbance we examined, captivity poses the most contrasting diet from the wild environment. Animals in captivity tend to have a simple and low-fiber diet, so as the captive individuals in the present study which feed mainly on commercial monkey chow. In addition to dietary change, other environmental factor relating to human disturbance that may be related to altered composition in gut microbiome, such as hygiene, home range, social contact, and geography (Clayton et al., 2016, 2018a). This is corroborated by the most distinctive gut microbial community of our captive individuals living in either cage or enclosure. Recent research comparing captive and wild mammals also detected a general pattern of composition shift in captive animals (Clayton et al., 2016, 2018a; McKenzie et al., 2017). These studies attributed the shifts to the reduced diet diversity and fiber intake. Compared to diet of captive individuals, diet of free-ranging individuals is more diverse and fiber-rich.

Our data set further supports that the gut microbiota of Japanese macaques is related to the specific diet macaques consumed under different disturbance types. For example, we found chloroplast abundance, an indirect indicator of plant intake, more enriched in macaques less disturbed by human activities. As reported in red-shanked doucs, abundance of chloroplast is also positively related to the wildness of doucs' lifestyle; chloroplast was barely observed in captive douc populations, while a considerable amount was detected in wild populations (Clayton et al., 2016). Difference in chloroplast abundance, therefore, may reflect the macaques' intake of fibrous food in different conditions. In PRI, captive macaques are fed predominantly with easily digestible monkey chow, and sometimes minor food items like sweet potatoes (Jaman & Huffman, 2008; Jaman et al., 2010). Every 100 g of monkey chow contains approximately 44.5 g soluble non-nitrogen matter, 28.2 g crude protein, 9.5 g crude lipid, 8.2 g water, 2.5 g crude fiber, and 2.5 g crude ash (Jaman et al., 2010). In contrast to that, the average NDF of major food leaves consumed by wild macaques were around 42% (Hanya, Kiyono, Takafumi, Tsujino, & Agetsuma, 2007). In the wild, such kinds of fiber-rich food is an important part of the macaques' daily diet (Hanya, 2004a, 2004b, 2010). Yakushima highland macaques spent 45% of annual feeding time on fiber-rich food items (Hanya, 2004a), and the lowland macaques spent around 35% (Hill, 1997). Presumably, the provisioned and crop-raiding macaques consume a mixed diet of agricultural crops and forest foods, with differing proportions between sites. Within provisioned samples, the diet of Koshima macaques resembles that of wild macaques since provisioning was restricted to twice a week (Go, 2010; Leca et al., 2008; Tsuji, Ito, Wada, & Watanabe, 2015). Relative to Koshima macaques, Shodoshima macaques are intensively provisioned by visitors and staff of the monkey park, for about three to four times per day (Leca et al., 2008). On the contrary, the diet of crop-raiding macaques is rarely studied. Some studies suggested that crop-raiding events were highly related with food availability in the forest; in food scarce seasons like summer and winter, macaques rely more on human settlements and crops (Ueda, Kiyono, Nagano, Mochizuki, & Murakami, 2018; Yamada & Muroyama, 2010). Our crop-raiding samples were collected in early July, hence the macaques may have fed on crops.

Another exemplary bacterial indicator revealing the relationship between diet and the gut microbiome may be the Firmicutes and Bacteroidetes ratio. A negative relationship was found between the Firmicutes and Bacteroidetes ratio and the availability of anthropogenic food for the macaques: the highest ratio was found in wild Japanese macaques, intermediate in crop-raiding and provisioned, and lowest in captive macaques. In our data set, the increasing abundance of Firmicutes microbes in wild macaques' gut was mainly accounted by microbes from families Lachnospiracea, Ruminococcaceae, and Peptococcaceae. In particular, microbes from Lachnospiracea and Ruminococcaceau play role as active plant degraders with identified key carbohydrate-active enzymes, sugar transport mechanisms, and metabolic pathways (Biddle, Stewart, Blanchard, & Leschine, 2013). A positive relationship between host fiber intake and the abundance of Lachnospiraceae and Ruminococcaceae was also found in Sifakas (Springer et al., 2017) and black howler monkeys (Amato et al., 2015a).

Interestingly within undisturbed populations from Yakushima, we also found a higher ratio in highland macaques which consume a large amount of fiber-rich food throughout the year (Hanya, 2004a). Similarly, within wild rhesus macaque populations, macaques from high altitude regions exhibited an elevated ratio possibly as an adaptation to fiber-rich diet and increased energy consumption in high altitude (Zhao et al., 2018). Hence, the ratio may be related to the fruit and fiber consumption of mammals, including Japanese macaques in this case, in different environments. Considering Firmicutes and Bacteroidetes are commonly present in most mammals, the ratio could be a suitable indicator for not only the macaques but also other wild animals. However, there is still no direct test for the causal relationship between the Firmicutes to Bacteroidetes ratio and fermentative ability of gut microbiome yet. For example, in vitro digestibility assay for testing fermentative capacity on same food item may be good option for further research.

Nevertheless, the Firmicutes to Bacteroidetes ratio does not completely mirror the trend for Chloroplast abundance, the indirect measure for fiber intake (Clayton et al., 2016, 2018a). For example, Koshima macaques, which had exceptionally enhanced abundance of Chloroplast, did not necessarily hold higher Firmicutes to Bacteroidetes ratio. It is possible that the Koshima macaques acquire chloroplast from other sources. Koshima macaques have been previously reported to use fish as a food source; the elevated ratio may be related to ingestion of herbivorous fish (Sullam et al., 2012; Watanabe, 1989). Nevertheless, our reasoning is limited as we did not collect detailed dietary data around the time of sample collection. To unravel the diet–gut microbiome relationship of Japanese macaques, further studies on the gut microbiome combining detailed dietary data are required.

4.3 | Effect of anthropogenic food availability on the gut microbiome of Japanese macaques: Contrasts with other species

Aside from similar patterns, we detected in Japanese macaques and other mammals, alpha diversity and relative abundance of some gut bacterial taxa of Japanese macaques showed unexpected response toward the inclusion of anthropogenic food in the diet. Comprehensive research of captive, semicaptive, and wild NHPs suggested that alpha diversity of NHPs' gut microbiota was significantly reduced by the dietary shift associated with provisioning (Clayton et al., 2016, 2018a). Although we did detect lower Shannon diversity index in captive than wild populations, both observed richness and Shannon diversity did not show a decreasing trend along with the anthropogenic food availability in the habitat. In terms of indicator bacterial taxa, our wild populations had higher abundance of genus *Bacteroides*, which was found more enriched in other humanized NHPs (Clayton et al., 2016; Jia et al., 2018).

These differential patterns in the macaque gut microbiome may be associated with the species-specific response of Japanese macaques to inclusion of anthropogenic food in daily diet. Host traits such as host taxonomy, foraging ecology, and gut physiology could result in deviating responses in gut microbiome even toward similar environmental stimuli (Amato et al., 2015b, 2016; McKenzie et al., 2017). In particular, host taxonomy plays an important role in determining the set of gut microbiome harbored by the species and thus response may vary across NHP species (Amato et al., 2015b; Ley et al., 2008a, 2008b; McCord et al., 2014; McKenzie et al., 2017). In studies encompassing 41 species of mammals across six orders, reduced alpha diversity of gut microbiome in captivity is not a universal phenomenon (McKenzie et al., 2017). Among the 11 mammalian families investigated, six families showed no significant change in alpha diversity, while four had significantly decreased and one had significantly increased diversity in captivity. And again, relative abundance of bacterial taxa changes in different ways with regard to the host species. Comparison between two closely related howler monkey species by Amato et al. (2016) also revealed that a small difference in host genetics could result in differential responses of the gut microbiome; despite sharing many microbial genera, mantled howler monkeys had gut microbiota more resistant to dietary shifts than black howler monkeys. Our study subject, the Japanese macaques, may be another example suited to human-disturbed habitats by having taxonomically diverse but functionally redundant microbes. In our study, the gut of captive macaques maintained gut microbial diversity similar to that of wild macaques, which may sustain macaques even in the suboptimal condition. It is possible that unrelated gut microbial taxa perform a similar function as a result of convergent evolution (Moya & Ferrer, 2016; Muegge et al., 2011). Consequently, specific bacterial taxa may vary in response due to the potential difference in cross-feeding and competition at lower taxonomic levels.

On top of host taxonomy, host foraging ecology and gut morphology could lead to species-specific response in gut microbiome. Compared with folivorous NHPs examined in previous studies, for example, red-shanked doucs (Clayton et al., 2016, 2018a) and black howler monkeys (Amato et al., 2013; Bennett et al., 2016), reliance of Japanese macaques on gut microbiome to digest the fiber-rich plant materials may not be as high. Though their diet contains fibrous food items, Japanese macaques are not strictly herbivorous but feed on more nutritious foods such as fruits and nuts whenever available (Hanya, 2004a). Indeed, some folivorous NHP species like doucs develop an enlarged gut for extended fermentation (Caton, 1998; Lambert, 1998), but this is not the case for Japanese

macaques which are caeco-colic/hindgut fermenters. For foregut fermenters, the food items arrive at the fermentation chamber undigested, leaving more nutrients available to the gut microbes. Opposite to that, caeco-colic/hindgut fermenters absorb all the digestible components from food before the fermentation. As gut morphology determines the host digestive physiology, even the same food item could have a different impact on the gut microbiome depending on the gut morphology of the hosts (Lambert, 1998).

4.4 | Limitations

In this study, we used the terms "availability of/accessibility to anthropogenic food" and "disturbance type" in categorizing and describing macaque populations. Such terms may be vague because the pattern we observed here is not solely attributed to diet, but the synergy of multiple environmental factors like geography, home range, and social interactions. For examples, captive populations generally had distinctive gut microbiota with other free-ranging populations. Aside from diet, the distinctive gut microbiota harbored by captive populations may be attributed to reduced contact with potential microbes due to limited home range and social interaction. Despite the presence of confounding factors, we believe that the dietary change caused by human disturbance is one of the major elements leading to the difference in gut microbiota presented in this study.

However, another problem in this study is that the sampling sites and populations experiencing different human disturbance level are confounded. For example, two groups for wild population were only gathered in Yakushima island, and captive samples were all from PRI only. The gut microbiota may be more similar to each other because they are from close site, not only because of the difference in disturbance level and anthropogenic food availability among sites. Again, we argue that the effect of sites is weak, though not completely negligible. If effect of sites were greater, then gut microbiota of both wild groups from Yakushima island should be more similar. Yet in our study, Yakushima lowland samples are in fact more similar to Koshima samples than to Yakushima highland. To unravel the diet–gut microbiome relationship of Japanese macaques, further studies including more sites is highly recommended.

In addition to the abovementioned points, we were not able to analyze possible sex and age effect. It is supported by multiple studies that individuals from different age-sex classes have different crop-raiding tendencies. Depending on age-sex class of the individuals, the quantity and types of anthropogenic food may be differentially consumed.

5 | CONCLUSION

Overall, our results demonstrated that it is possible to predict animals' degree of reliance on anthropogenic food through gut microbiota, but one should always pay attention to species-specific response of the animals' gut microbiota. Even genetically closely related species could exhibit distinctive responses due to a species trait, severity of disturbance, and characteristics of gut microbes. This suggests that the picked bacterial taxa in this study may only be applicable to Japanese macaques but not other species. To some degree, the gut microbiome can provide a general picture of human disturbance as our data did reveal a gradual change of gut microbial community along anthropogenic food availability in macaques' habitat. Despite that, one should consider carefully if alpha diversity and relative abundance of certain gut microbial taxa can be used in assessment. Additionally, the differential responses exhibited by Japanese macaques may also mean that more cautions should be taken when using NHP models for inferring the host–gut microbiome relationship of humans. This is especially true when considering unique human physiological adaptations and dietary shifts across evolutionary time, which may lead to further deviation of response in the gut microbiome.

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OPEN RESEARCH BADGES

This article has been awarded Open Materials and Open Data badges. All materials and data are publicly accessible via the IRIS Repository at https://www.iris-database.org/iris/app/home/detail?id=york:934328.

DATA AVAILABILITY STATEMENT

The data have been deposited in the DDBJ database with accession number DRP005397.

ORCID

Wanyi Lee http://orcid.org/0000-0001-8610-9805
Goro Hanya http://orcid.org/0000-0002-8612-659X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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