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Dietary and Sexual Correlates of Gut Microbiota in the Japanese Gecko, *Gekko japonicus* (Schlegel, 1836)

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Simple Summary: The structure and composition of gut microbiota are usually influenced by the host sex and diets. We used wild-caught Japanese geckos (*Gekko japonicus*) and captive conspecifics fed with specific food in the laboratory to study the differences in gut microbial structure and composition of geckos. The composition of Japanese gecko gut microbiota is more similar to leopard geckos rather than to other groups in reptiles. Diet and sex differences could shape the gut microbial structure and composition in geckos.

Abstract: Numerous studies have demonstrated that multiple intrinsic and extrinsic factors shape the structure and composition of gut microbiota in a host. The disorder of gut microbiota may trigger various host diseases. Here, we collected fecal samples from wild-caught Japanese geckos (*Gekko japonicus*) and captive conspecifics fed with mealworms (mealworm-fed geckos) and fruit flies (fly-fed geckos), aiming to examine dietary and sexual correlates of gut microbiota. We used the 16S rRNA gene sequencing technology to determine the composition of gut microbiota. The dominant phyla with a mean relative abundance higher than 10% were Verrucomicrobiota, Bacteroidota and Firmicutes. Gut microbial community richness was higher in mealworm-fed geckos than in fly-fed and wild geckos, and community diversity was higher in mealworm-fed geckos than in wild geckos. Neither alpha nor beta diversity of gut microbiota differed among wild, mealworm-fed and fly-fed geckos. The beta rather than alpha diversity of gut microbiota was sex-dependent. Based on the relative abundance of gut bacteria and its gene functions, we concluded that gut microbiota contributed more significantly to the host's metabolic and immune functions. Higher diversity of gut microbiota in mealworm-fed geckos could result from higher chitin contents of insects of the order Coleoptera. This study not only provides basic information about the gut microbiota of *G. japonicus*, but also shows that gut microbiota correlates with dietary habit and sex in the species.

Keywords: ASVs; diet habit; *Gekko japonicus*; gut microbiota; sex

1. Introduction

Gut microbiota is known as the second genome of the host [1], encoding the 10-100 times the number of genes of the host genome [2]. Gut microbiota plays a key role in host survival and adaptation, with its functions mainly manifested in a host's life history [3], physiology [4], immune [5], growth [6], development [4] and behavior [7]. Gut microbiota can change rapidly in response to changes in the host's environmental conditions and dietary habits [8], induce a host's metabolic flexibility and phenotypic plasticity, and therefore enhance its ability to adapt to the environment [3].

For example, taxonomical shifts in gut bacterial communities in juvenile ostriches (*Struthio camelus*) coincide with the cessation of yolk absorption, co-occurring with their dietary switch [6]. These shifts may help ostriches adapt to dietary changes. For example, short chain fatty acids produced by the gut microbiota can maintain gut homeostasis [9]. Gut microbial dysbiosis can induce various host diseases and even threaten host survival [10].

The structure and diversity of gut microbiota are susceptible to numerous external and internal factors, including the host's taxonomic category [11,12], sex [13], healthy status [14], age [6], dietary habit [15] and living environment [16]. These factors can substantially influence the composition, abundance and diversity of gut bacterial communities. In lizards, for example, captivity changes the gut microbial composition in *Shinisaurus crocodilurus* [17], *Takydromus septentrionalis* [18], and *Tremarctos ornatus* [19]. Toad-headed lizards (*Phrynocephalus vlangalii*) from the highest-altitude population have the lowest gut microbial diversity [16]. On the contrary, Glires mammals from high-latitude regions have a higher gut microbial diversity than their low-latitude conspecifics, because the increased energy demands in cold and hypoxic environments cannot be met without increasing gut microbial diversity [20].

The gut microbial composition is largely host-taxa specific [12]. In invertebrates, for example, the dominant gut microbial phyla are Tenericutes, Firmicutes, and Proteobacteria in snails [21,22], and Proteobacteria and Firmicute in insects [23]. In vertebrates, the dominant gut microbial phyla are Firmicutes and Bacteroidetes in amphibians [24], reptiles [25], and mammals [12], and Proteobacteria and Firmicutes in birds [26]. It is of great significance to explore the factors affecting gut microbiota and host-microbe symbiotic relationships. One widely accepted idea is that diet and host genetic status have a key role in shaping gut microbial structures [12,23].

Each microbial taxon has its functional roles in the host gut. Bacteria of the phylum Bacteroidetes are the homeostasis cornerstone in a health gut and involve in various functions, including the gut-brain-axis interactions, the immune system and metabolic homeostasis [27]. A large number of genes of the phylum Firmicutes are clustered to encode the ABC-type sugar transport systems, and bacteria of this phylum usually are active in carbohydrate metabolism [28]. Therefore, the ratio of Bacteroidetes to Firmicutes in relative abundance is correlated with the slim figure, with a higher ratio hinting a healthier host, which in turn correlates with host obesity [29]. Proteobacteria is regarded as a potential diagnostic signature of dysbiosis and risk of disease in human [28], but the ratio Proteobacteria to Firmicutes and Bacteroidetes in relative abundance is correlated with the bacterial stress tolerance under cold environments [30]. It is the role of gut microbiota that assists the host to adapt to a wide variety of diets and environments.

Reptiles are the first group of vertebrates that can truly live out of water on land and their gut bacterial variation has therefore attracted much attention. As in other animal taxa, gut microbiota is affected by many factors in reptiles, including host genetic status [25], captivity [18], environment [16], and diet [17]. Previous studies on reptiles have showed that a given factor may affect the gut microbiota in some species but not in others. For instance, diet shapes gut microbiota in *S. crocodilurus* [17] but not in *Varanus salvator* [31]. Compared with other reptile taxa, studies on gut microbiota in geckos have been limited, focusing only on the effects of fasting on gut microbiota in *Eublepharis macularius* [32,33] and the structure of gut microbiota in *Hemidactylus frenatus* [34]. Here, we used high-throughput sequencing to study dietary and sexual correlates of the gut microbiota in the Japanese gecko, *Gekko japonicus*. This gecko is a small-sized, oviparous species of the family Gekkonidae, occurring in the central and southeastern parts of China, Japan and Korea. The gecko is a comparatively well-known lizard species in China, with data collected over the past few years covering a wide range of topics such as genomics [35], temperature-dependent sex determination [36,37], molecular basis of character development [38], and microhabitat use [39].

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2. Materials and Methods

2.1. Sample Collection

We used 49 adult geckos without any signs of disease (including ectoparasites) to conduct this study. All these geckos were collected in Xianlin Campus of Nanjing Normal University (NNU), 25 (14♀♀and 11♂♂) in June 2020 and 24 (11♀♀ and 13♂♂) in September 2020. Geckos collected in June were individually housed in 175 × 175 × 152 mm (length × width × height) plastic cages placed in a room where temperatures varied naturally. Of the 25 geckos, 13 (7♀♀and 6♂♂; hereafter mealworm-fed geckos) were fed with mealworms (larvae of Tenebrio molitor), and 12 (7♀♀ and 5♂♂; hereafter fly-fed geckos) with fruit flies (Drosophila melanogaster), both for three months, during which period distilled water was available ad libitum. All facilities were disinfected by wiping with 97% alcohol every other day. Mealworm- and fly-fed geckos always had free access to food sterilized with UV light for 1 h in advance. Geckos collected in mid-September (hereafter wild geckos) were individually housed in sterile 175 × 175 × 152 mm cages overnight and then collected fecal samples. In September, we used light traps to collect insects at the sites where we collected geckos, thereby assessing prey items potentially available to geckos in the wild. Insects of the orders Lepidoptera and Diptera were the most abundant prey items potentially available to Japanese geckos in Xianlin Campus of NNU (Table 1).

Table 1. Prey items potentially available to Japanese geckos in the wild.

Abundance of prey items	Order
Numerous (> 500)	Lepidoptera, Diptera
More (between 100 and 500)	Coleoptera, Hemiptera
Medium (between 50 and 100)	Hymenoptera, Ephemeroptera, Trichoptera
Fewer (between 10 and 50)	Orthoptera, Mantodea, Neuroptera, Megaloptera, Thysanoptera, Plecoptera, Blattodea
Least (< 10)	Dermaptera, Odonata, Corrodentia, Rhaphidioptera

[1] The abundance of prey items is sorted by the number of insects found in the light trap.

We put fecal samples collected from mealworm-fed, fly-fed and wild geckos into sterile tubes, labeled these tubes, and then stored them at -20 °C for late DNA extraction. We released all geckos at their point of capture soon after the collection of fecal samples in mid-September. Geckos of different groups did not differ from each other in mean values for body mass ($H_{2,49} = 1.95$, $p = 0.38$) and snout-vent length ($H_{2,49} = 2.62$, $p = 0.27$). Our experimental procedures complied with laws on animal welfare and research in China, and were approved by the Animal Research Ethics Committee of Nanjing Normal University (Permit No. IACUC 20200511).

2.2. DNA Extraction, PCR Amplification and Sequencing

We used the Mag-Bind Soil DNA Kit (Omega, Shanghai, China) to extract the microbial DNA from the fecal samples according to the manufacturer protocols. We used 2.0% agarose gel electrophoresis and Qubit 3.0 DNA detection kit (Thermo Fisher Scientific) to purify and quantify the DNA products, respectively. The bacterial V3-V4 region of the 16S rRNA gene were amplified using PCR with a 30 μL reaction system including 15 μL of 2× Hieff® Robust PCR Master Mix (2×), 1 μL of each primer (10 μM), 20 ng of genomic DNA, and ddH_2O. The universal primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) were selected to perform the PCR reaction. The first round of PCR thermal cycling conditions was performed as follows: initial denaturation at 94 °C for 3 min, followed by 5 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 20 s and extension at 65 °C for 30 s. The other 20 cycles consisted of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. In the second round, PCR products of the first round were used for amplification, and llumina bridge PCR compatible primers were introduced. The PCR reaction system was the same as the first round. The thermal

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cycling conditions were as follows: denaturation at 95 °C for 3 min, followed by 5 cycles of denaturation a 94 °C for 20 s, at 55 °C for 20 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Sequencing of the PCR-amplified products was conducted on an Illumina MiSeq.

2.3. Quality Control and Data Standardization

We imported the raw paired-end sequence into Quantitative Insights into Microbial Ecology 2 (QIIME2) using the manifest file and trimmed the primers [40]. We used the DADA2 to filter and truncate low-quality reads and produce paired-end reads [41]. These reads after quality control were generated the raw amplicon sequence variants (ASV) with a minimum overlap of 12 bp. The raw paired-end sequences were submitted to the National Genomics Data Center (NGDC) GSA database (accession number CRA007161).

We used QIIME2 to classify ASVs into organisms based on pre-formatted SILVA 138 SUU NR99 ASVs full-length reference sequences following the q2-fragment-classifier method in QIIME2. The sequencing depth for each sample was calculated using QIIME2 and visualized using R 4.0 [42]. We removed ASVs with the number less than 10 in only one sample for further analysis to avoid large partial sample deviations. The abundance information was standardized based on the sample with the least ASVs number.

2.4. Estimation of Alpha and Beta Diversity

We used QIIME2 to calculate alpha diversity indexes, including community richness (observed species), community diversity (Shannon's entropy index), and community evenness (Pielou's evenness index). We used Kruskal-Wallis H and Mann-Whitney U test to examine whether alpha diversity indexes differed between (mealworm-fed, fly-fed and wild) gecko groups and between sexes, respectively. Pairwise comparisons using Wilcoxon rank sum test with continuity correction were performed when necessary. For beta diversity, we used principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (Adonis) to show differences in microbial community structure among gecko groups. Adonis was performed based on the Bray-Curtis distance with 999 permutations. The linear discriminant analysis of effect sizes (LEfSe) and linear discriminant analysis (LDA) were conducted to compare the microbial abundances from the phylum to genus levels based on the relative abundance higher than 1% [43]. The unique bacterial taxa were determined based on log LDA score > 2 and $p < 0.05$. Kruskal-Wallis H test was used to verify whether the bacteria detected by LDA had a higher relative abundance among the different diet × sex combinations.

2.5. Gene Function Predication

PICRUST2 was used to explore gene functions of all ASVs in gut microbiota based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [44]. We allocated these gene functions to the corresponding KEGG pathways and obtained KEGG Orthology (KO) information for each gene function for three KEGG pathways [45]. The relative abundance of these gene functions for each sample was calculated to assess the functional differences in gut microbiota among different gecko groups. The LEfSe and LDA were performed to compare the relative abundance of KEGG gene functions from level 1 to level 3 based on the relative abundance higher than 1%. Only the gene functional category with a log LDA score > 2 and $p < 0.05$ was used in this analysis. Kruskal-Wallis H test was used to verify whether the gene function detected by LDA had a higher relative abundance among the different diet × sex combinations. The unique and shared gene functions were visualized using Venn diagram. All values were presented as mean ± standard error (SE), and the significance level was set at $\alpha < 0.05$.

3. Results

We obtained 4,299,671 raw reads and 2473062 high-quality reads from the 49 fecal samples (Table A1). The number of observed bacterial ASVs firstly increased with the increase of the number

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of sequences and then leveled out in each sample (Figure A1). We identified 976 bacterial ASVs, with 114–214 ASVs per sample (Table A2). These ASVs could be allocated to 12 phyla, 19 classes, 49 orders, 83 families, and 168 genera.

The top four dominant bacterial phyla were Verrucomicrobiota (36.6 ± 3.5%), Bacteroidota (29.4 ± 2.2%), Firmicutes (18.9 ± 2.2%), and Proteobacteria (9.6± 2.3%) (Figure 1A). The dominant bacterial families with a relative abundance > 3% were Akkermansiaceae (35.3 ± 3.5%), Bacteroidaceae (18.0 ± 1.6%), Tannerellaceae (8.1 ± 1.0%), Enterobacteriaceae (6.2 ± 1.6%), Lachnospiraceae (4.4 ± 0.8%) and Clostridiaceae (4.3 ± 1.0%) (Figure 1B). The dominant genera with a relative abundance > 3% were *Akkermansia* (35.3 ± 3.5%), *Bacteroides* (18.0± 1.6%), *Parabacteroides* (5.7 ± 0.7%), and *Clostridium_sensu_stricto_1* (4.3 ± 1.0%) (Figure 1C).

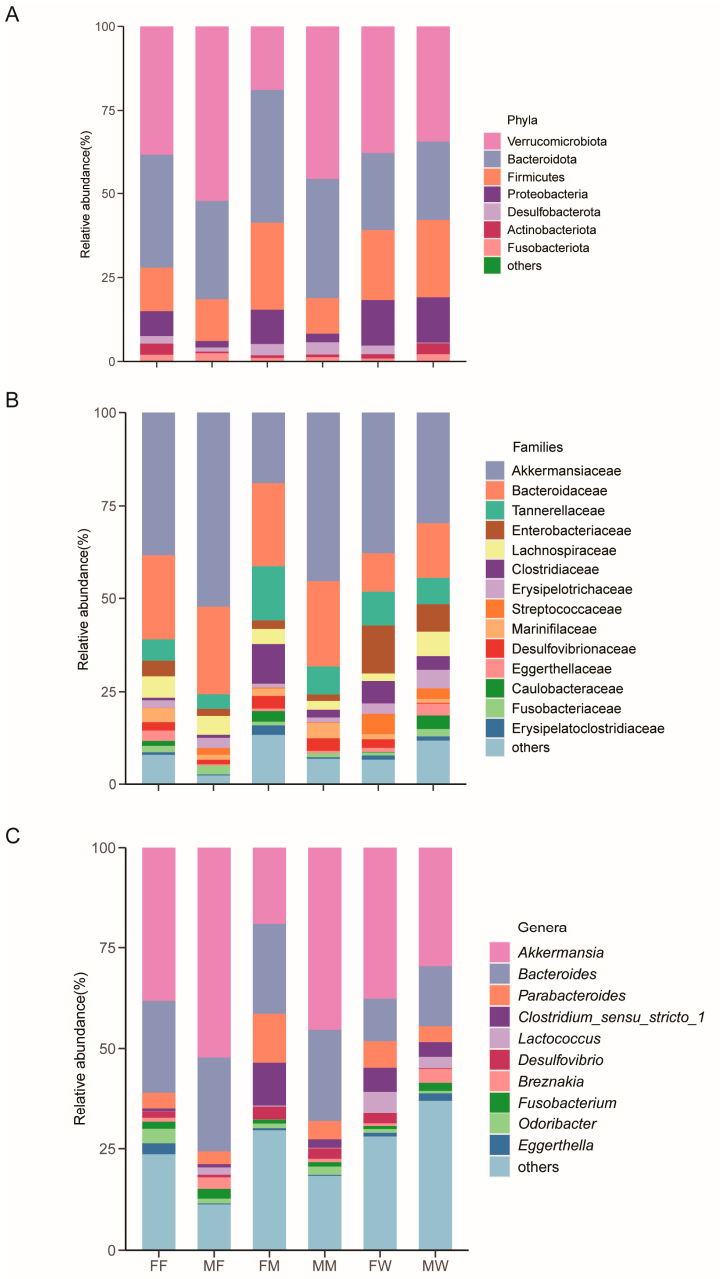

Figure 1. The relative abundance of the gut microbiota in each gecko group at the phylum (A), family (B), and genus (C) levels. Each color in a plot represents a taxonomic group, of which the name is shown on the right side of the plot. The color for 'others' indicates all other phyla (A), families (B), or genera (C) combined, of which the names are not listed in each plot. FF: fly-fed females; fly-fed males; FM; mealworm-fed females; MM: mealworm-fed males; FW: wild females; MW: wild males.

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3.2. Dietary and Sexual Correlates of Gut Microbiota

Kruskal-Wallis showed that community diversity (H= 7.80, df=2, p = 0.02) and richness (H = 7.53, df=2, p = 0.02) rather than community evenness (H = 5.93, df=2, p = 0.05) differed among mealworm-fed, fly-fed and wild geckos. Specifically, gut microbial community richness and gut microbial community diversity were significantly higher in mealworm-fed geckos than in wild geckos (Figure 2). None of the above three diversity indexes differed between the sexes (all p > 0.05) (Figure 2).

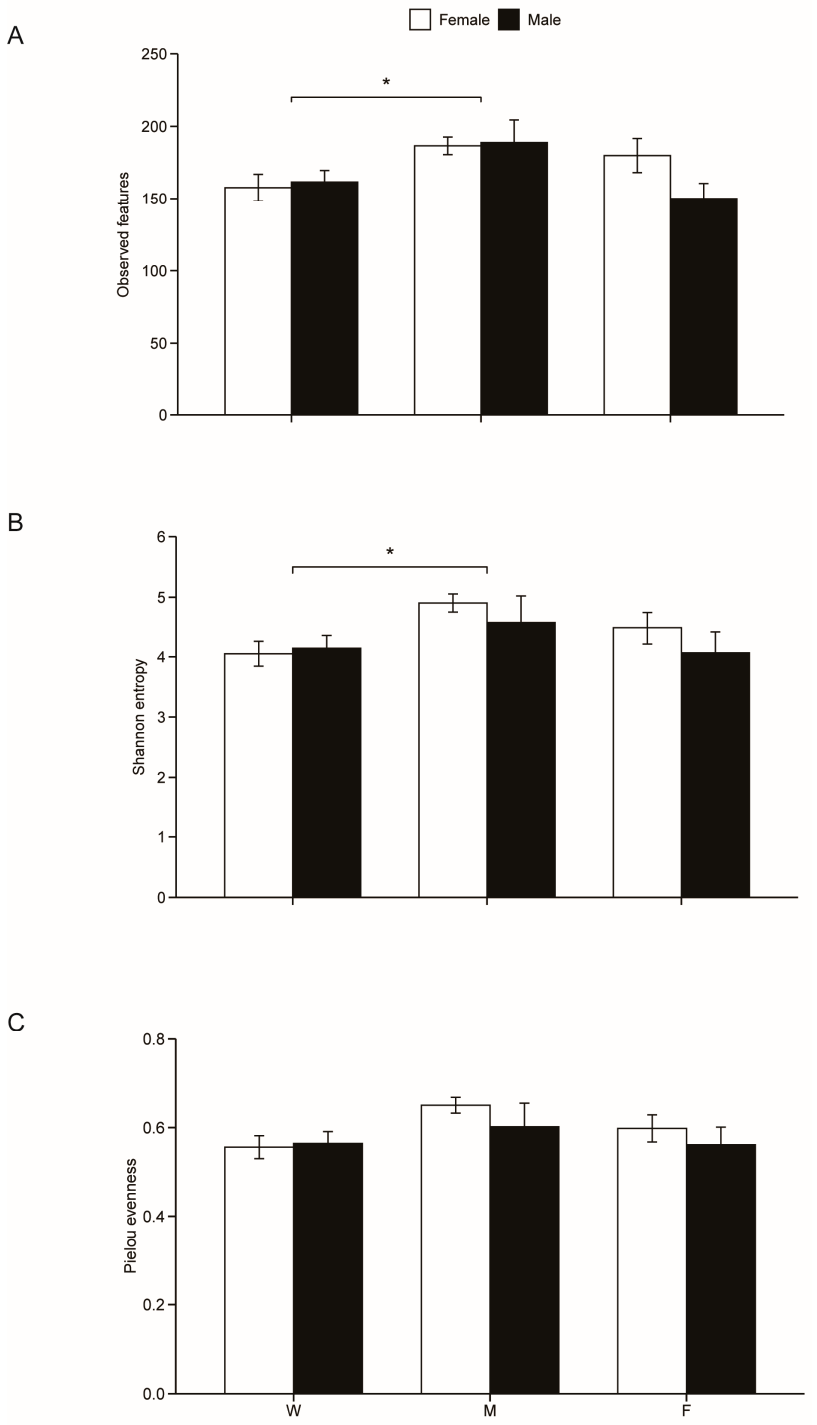

Figure 2. The alpha diversity indexes of gut microbiota in six diet × sex combinations of fecal samples, including observed species (A), Shannon's entropy index (B) and Pielou's evenness index (C). F for fly-fed geckos, M for mealworm-fed geckos, and W for wild geckos.

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The PCoA based on the Bray-Curtis distance showed a significant separation of gut microbiota among six diet × sex combinations (Adonis: $r^2 = 0.15$, $F_{5,43} = 1.46$, $p = 0.006$), with the first and second axes respectively explaining 16.9% and 11.2% of the total variance (Figure 3A). However, the significant separation of gut microbiota was be found only between the sexes (Adonis: $r^2 = 0.06$, $F_{1,47} = 2.89$, $p = 0.001$; Figure 3A), rather than in different diet groups (Adonis: $r^2 = 0.05$, $F_{1,46} = 1.21$, $p = 0.174$; Figure 3B). In addition, neither in males (Adonis: $r^2 = 0.09$, $F_{2,21} = 1.10$, $p = 0.32$; Figure 3C) nor in females (Adonis: $r^2 = 0.09$, $F_{2,24} = 1.11$, $p = 0.30$; Figure 3D) did gut microbiota differed among mealworm-fed, fly-fed and wild geckos. LEfSe analysis showed significant differences in the unique gut microbiota among fly- and mealworm-fed females, mealworm-fed males, and wild males (Figure 4). Specifically, the unique bacteria family Desulfovibrioria and Marinifilaceae was found in mealworm-fed males, the families Eggerthellaceae and Caulobacteraceae was unique in wild males, the unique bacteria genera *Eggerthella*, *Bacteroides* and *Odoribacter* was found in fly-fed females, and the family Erysipelatoclostridiaceae and Tannerellaceae, and genera *Desulfovibrio* and *Clostridium_sensu_stricto_1* was unique in mealworm-fed females (Figure 4). Kruskal-Wallis H test showed that the relative abundance of above bacterial taxon had significant differences among different groups except for the family Tannerellaceae (Table A3).

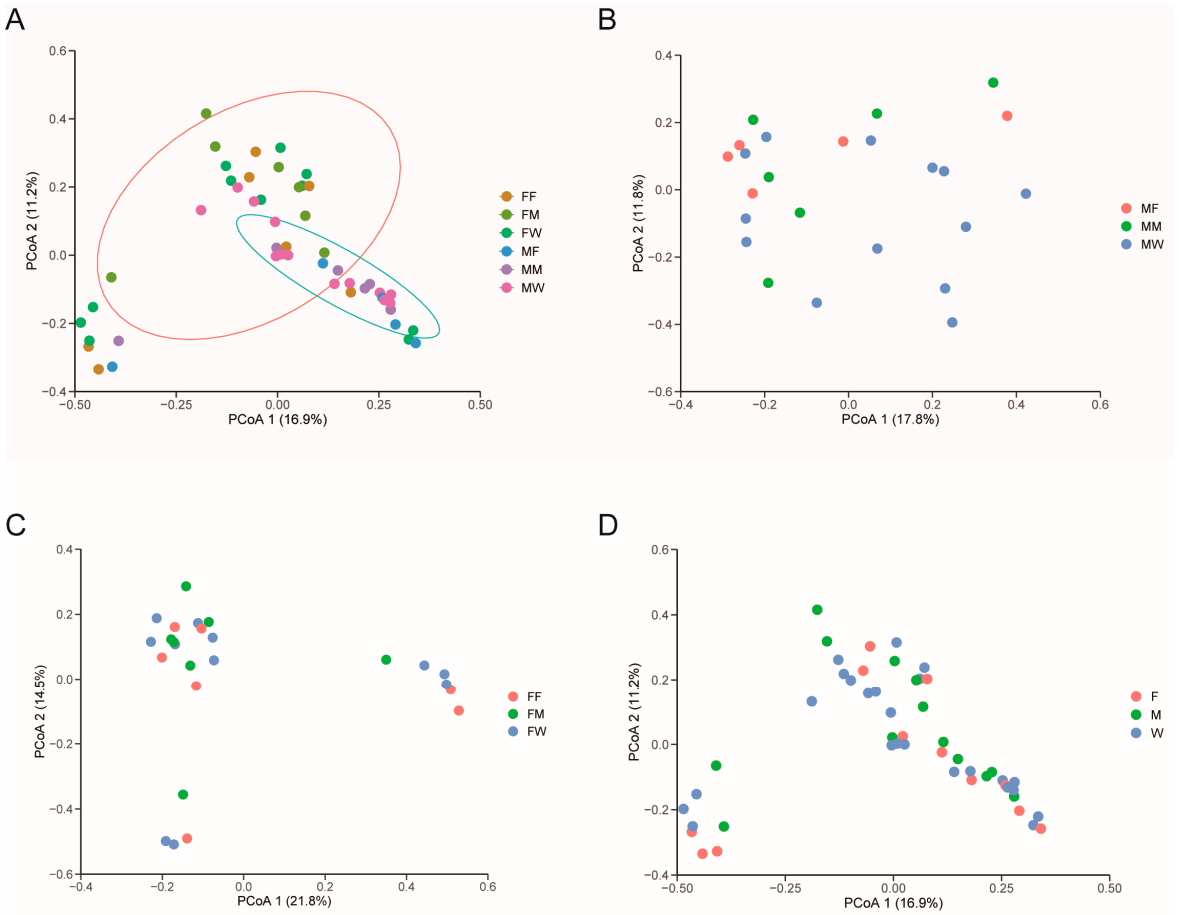

Figure 3. Gut microbial diversity in six diet × sex combinations of fecal samples (A), fecal samples from three different diet group (B), male (C) and female (D) geckos ingesting different prey items. Principal coordinates analysis of Bray-Curtis distance matrix for bacterial community diversity. See Figure 1 for the definition of each group.

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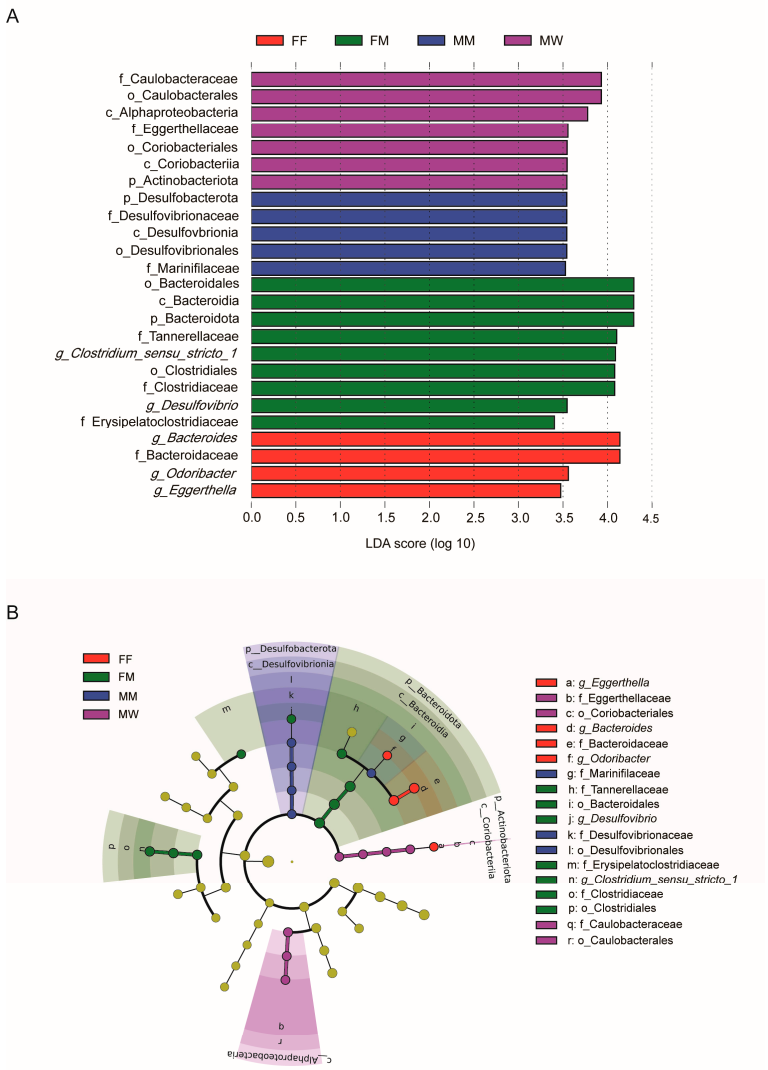

Figure 4. Differences in gut microbiota among the four groups are determined by LEfSe (A). LDA scores reflect the differences in relative abundance among the four groups (B). See Figure 1 for the definition of each group. The letters "o", "f" and "g" indicate order, family and genus, respectively.

3.2. The Predicted Metagenomes

The predicted functions in gut microbiota were mainly involved in metabolism (80.8 ± 0.2%), genetic information processing (12.8 ± 0.2%), cellular processes (3.2 ± 0.1%), environmental information processing (2.4 ± 0.1%), organismal systems (0.4 ±0.01%), and human diseases (0.32 ± 0.02%) at the first level (Figure 5A). The second KEGG category level was composed of 31 functions, among which the most abundant categories with a relative abundance > 5% in gut microbiota had functions associated with carbohydrate metabolism (15.0 ± 0.1%), metabolism of cofactors and vitamins (13.5 ± 0.2%), amino acid metabolism (12.2 ± 0.1%), metabolism of terpenoids and polyketides (8.9 ± 0.1%), glycan biosynthesis and metabolism (6.9 ± 0.2%), metabolism of other amino acids (6.8 ± 0.1%), lipid metabolism (6.1 ± 0.1%), replication and repair (5.9 ± 0.1%) and energy metabolism (5.3 ± 0.05%) (Figure 5B). Among 157 KEGG functions at the third level, those with a relative abundance > 2% were biosynthesis of ansamycins (3.7 ± 0.1%), other glycan degradation (2.7 ± 0.1%), biosynthesis of vancomycin group antibiotics (2.6 ± 0.1%), valine, leucine and isoleucine biosynthesis (2.1 ± 0.02%) (Figure 5C).

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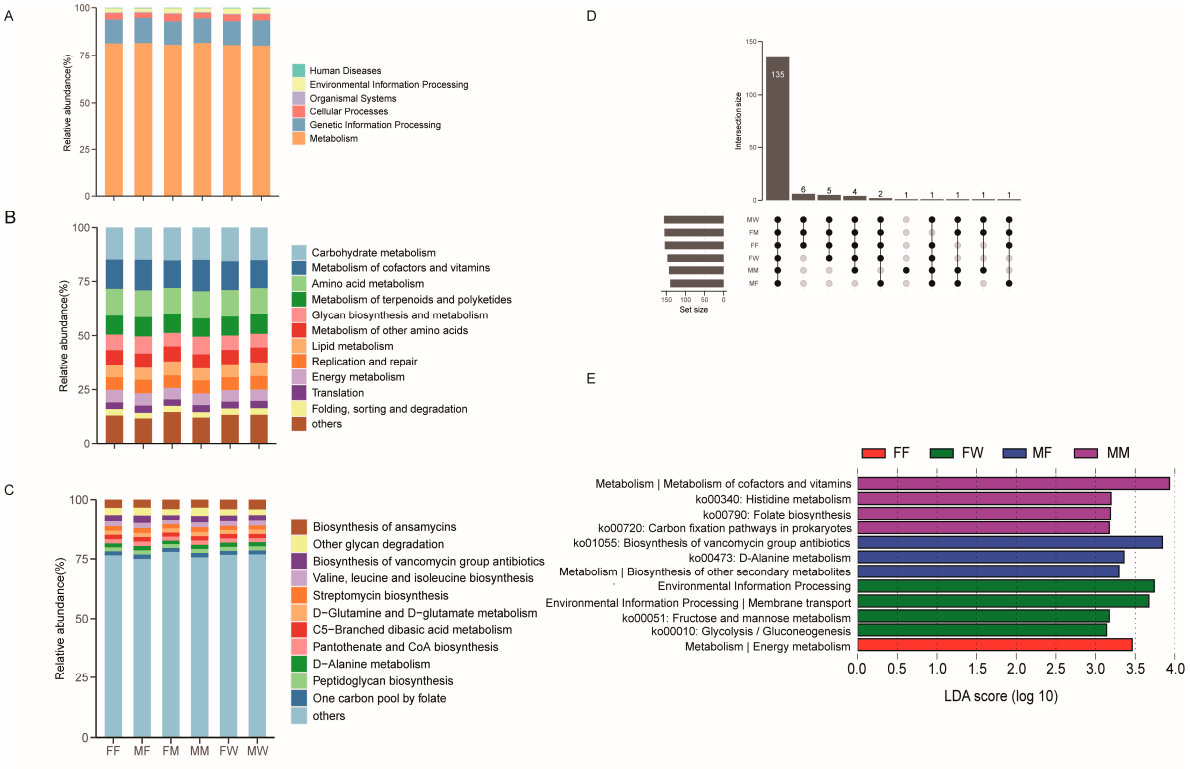

Figure 5. The relative abundance of gene functional categories based on 16S RNA in the gut microbiota at top (A), second (B) and third (C) levels, and the Venn diagram of functional gene among the diet and gender combine groups (D). LDA scores reflect the differences in relative abundance among among the diet and gender combine groups (E). Each color in a plot indicates one gene function. Detailed descriptions are shown on the right side of each plot. The colors for others in plots B and C indicate all other gene functions not listed in these two plots. See Figure 1 for the definition of each group.

A total of 157 known KO functional genes were identified. Geckos in six diet × sex combinations shared 136 genes (Figure 5D). LEfSe analysis based on KOs revealed that an unique gene function related to energy metabolism was found in fly-fed females (Figure 5E). In wild females, gene functions related to carbohydrate metabolism (Ko00010 and Ko00051) and environmental information processing and membrane transport were unique (Figure 5E). Gut microbial functions in fly-fed males had three unique functions related to metabolism (Ko00473, Ko01055, and biosynthesis of other secondary metabolites; Figure 5E). Gut microbial gene functions in mealworm-fed males were mainly associated with metabolism (Ko00340, Ko00720, Ko00790, and metabolism of cofactors and vitamins; Figure 5E). Kruskal-Wallis H test showed that the relative abundance of above unique gene functions had significant differences among different groups (Table A4).

4. Discussion

At the phylum level, the dominant gut microbes in *G. japonicus* were Verrucomicrobiota, Bacteroidota, Firmicutes, and Proteobacteria (Figure 1A), This is consistent with what has been observed in leopard geckos (*Eublepharis macularius*) [32], but differs from the results reported for other reptilian taxa. For example, the dominant gut microbial phyla are Proteobacteria, Bacteroidetes, and Firmicutes in lizards [16,31,46] and snakes [47,48], Bacteroidetes and Firmicutes in turtles [25,49], and Fusobacteria, Proteobacteria, Firmicutes, and Bacteroidetes in crocodiles [50,51]. This indicates that the dominant gut microbial phyla differ among animal taxa. In fact, even among animals of the same evolutionary clade, their gut microbiota may differ significantly. For example, the dominant gut microbial phyla differ significantly between two species of turtles [25] and among four species of

snakes [47] reared under the same conditions. This inconsistency between species provides evidence for the genetic correlates of gut microbiota in reptiles.

Taxonomically, all gut dominant genera and families in *G. japonicus* belong to the four dominant phyla mentioned above. The members of the phylum Verrucomicrobiota are correlated with mucin-degrading, glucose homeostasis and inducing regulatory immunity [52], as well as reducing obesity risk [53]. Bacteria of the phylum Bacteroidota have functional roles in degrading the high molecular weight organic matter, activating T-cell mediated responses and producing butyrate to maintain a health gut [54]. Many studies have showed that bacteria of the phylum Firmicutes contribute to degrading complex carbohydrates of both plant and hosts [55]. Members of the phylum Proteobacteria are related to degrading and fermenting the complex sugars and producing the vitamins for their hosts [56].

There has been evidence that gut microbial compositions are closely correlated with food ingested by hosts [31] and with their sex [22]. In this study, mealworm-fed geckos had higher gut microbial community diversity and richness although diet diversity was higher in wild geckos (Figure 2). That food diversity is not associated with gut bacterial alpha diversity in *G. japonicus* is similar to the findings demonstrated in *V. salvator* [31], *Anser anser* [57], and *Ochotona curzoniae* [58]. However, there are some species such as *Gasterosteus aculeatus* and *Perca fluviatilis* [59] and *Fejervarya limnocharis* [60] where gut microbial alpha diversity is negatively correlated with diet diversity. Gut microbial alpha diversity did not differ between the sexes in *G. japonicus*, similar to the result reported for a wide range of vertebrates including fish [61], amphibians [62], birds [57], and mammals [63]. However, sexual differences in gut microbial diversity do exist in many animals, also including fish [64], birds [65], and mammals [66]. Taken together, available data show that dietary and/or sexual correlates of host gut microbial alpha diversity are species- or taxon-specific.

Sex rather and diet shaped beta diversity of the gut microbiota in *G. japonicus* (Figures 3 and 4). However, PCoA showed that gut microbial structure differed only between sexes, but not among mealworm-fed, fly-fed and wild geckos (Figure 3). LEfSe showed that gut bacterial relative abundance differed not only between the sexes but also among three groups of geckos ingesting different prey items (Figure 4). Bacteria of the families Eggerthellaceae and Caulobacteraceae were enriched in wild males. Eggerthellaceae bacteria play an import in the transformation of bioactive secondary plant compounds in human feces [67], and Caulobacteraceae bacteria actively metabolize linear alkylbenzene sulfonates in soil [68]. The enrichment of bacteria of the genera *Bacteroides*, *Eggerthella* and *Odoribacter* in fly-fed females was correlated with metabolism [69], polysaccharide degradation [70] and immune [71], respectively. A higher relative abundance of Erysipelatoclostridiaceae at the family level, and *Desulfovibrio* and *Clostridium_sensu_stricto_1* at the genus level in mealworm-fed females also was enriched. Bacteria of Bacteroidales [72], *Desulfovibrio* [55] and *Clostridium_sensu_stricto_1* [73] could contribute to metabolism, and members of Erysipelatoclostridiaceae play a role in immunity in the host gut [74]. Therefore, the differences in relative abundance of gut microbiota may contribute more to metabolic and immune functions in the gecko.

The gut microbial functions in *G. japonicus* were mainly related to metabolism at the first function level with a relative abundance > 80%, the metabolism-related function, replication and repair at the second level, antibiotic and partial amino acid biosynthesis, other glycan degradation at the third level with higher relative abundances (Figure 5). Gut microbial functions in most animals are closely related to metabolism, including fish [61], amphibians [75], reptiles [18], birds [76] and mammals [19]. Therefore, the gut microbiota plays an important role in host energy metabolism. This is also evidenced by the enrichment of gene functions with high relative abundance in different diet × sex combinations in *G. japonicus* (Figure 5). For example, a higher relative abundance of gene functions related to metabolism were enriched in all male geckos, fly-fed female and wild female geckos.

Prey items potentially available for the Nanjing population of *G. japonicus* in September consisted of insects of the orders Lepidoptera and Diptera (Table 1). Insects mainly contain protein (30–70% of dry mass), fat (~35% of dry mass), minerals and vitamins [77], and can modulate the gut

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microbiota and improve host health status [78]. Fruit flies and mealworms used in this study belong to the orders Diptera and Coleoptera, respectively. This might be the main reason for why the gut microbiome of fly-fed geckos was closer to that of wild geckos. However, mealworm-fed geckos fed on mealworm containing more chitin. Chitin is one of the most abundant biopolymers in nature [79] and can restore the compositional balance of the bacterial community [78,80]. In this study, more diverse gut bacteria in mealworm-fed geckos might result from abundant chitin in diets. Therefore, the gut bacterial alpha diversity in *G. japonicus* might be correlated with the type of insect diets.

5. Conclusions

Gut microbial community diversity and richness rather than community evenness differed among mealworm-fed and wild geckos. Gut microbial community richness and diversity were significantly higher in mealworm-fed geckos than in wild geckos. None of the above three diversity indexes differed between the sexes. There was a significant separation of gut microbiota between the sexes. Such a separation of gut microbiota did not exist among geckos ingesting different prey items in both sexes. The relative abundance of unique gut bacteria and gene functions differed among different diet × sex combinations. Our study demonstrated dietary and sexual correlates of gut microbiota in a gecko species.

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Institutional Review Board Statement: The work was carried out in compliance with laws on animal welfare and research in China, and approved by the Animal Research Ethical Committees of Nanjing Normal University (Approval number: IACUC-20200511).

Informed Consent Statement: Not applicable.

Data Availability Statement: All 16S rRNA gene sequences obtained in this study have been deposited in the National Genomics Data Center (NGDC) GSA database (accession number CRA007161).

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A

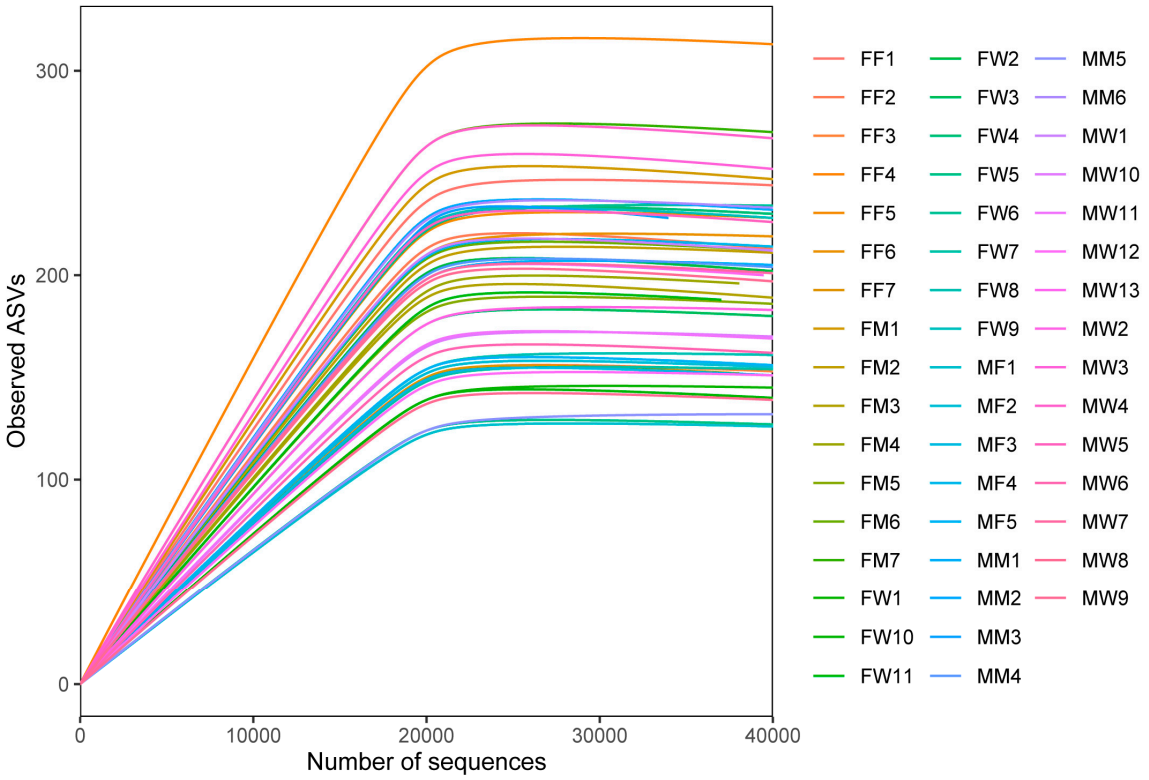

Figure A1. Rarefaction curves based on ASVs for individual fecal samples. Each color represents a sample.

Table A1. The number of valid reads and sequences information in each fecal sample.

Sample ID	Group	Raw reads	High-quality reads	Average sequence length	Minimum sequence length	Maximum sequence length	Accession number
FF1	FF	73684	40053	406.59	258	422	SAMC798099
FF2	FF	88949	60703	406.61	257	422	SAMC798104
FF3	FF	90123	51799	408.68	395	422	SAMC798108
FF4	FF	94885	66593	407.10	395	422	SAMC798112
FF5	FF	88354	44352	406.84	259	422	SAMC798130
FF6	FF	87440	53660	407.19	260	422	SAMC798131
FF7	FF	73619	38059	411.72	395	422	SAMC798132
FM1	FM	75766	41882	405.6	258	422	SAMC798096
FM2	FM	102760	70196	408.12	395	422	SAMC798098
FM3	FM	72769	40632	409.31	395	422	SAMC798101
FM4	FM	67705	38056	408.60	260	422	SAMC798103
FM5	FM	74409	43685	403.84	261	422	SAMC798105
FM6	FM	72310	43249	409.58	395	422	SAMC798107
FM7	FM	81193	59389	407.66	259	422	SAMC798110
FW1	FW	112652	66225	406.46	259	422	SAMC798095
FW2	FW	86098	43304	409.95	395	422	SAMC798097
FW3	FW	76989	41571	411.82	395	422	SAMC798100
FW4	FW	84867	46340	404.12	261	422	SAMC798102
FW5	FW	69268	42933	409.83	259	422	SAMC798106
FW6	FW	150444	86689	401.75	260	422	SAMC798109

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FW7	FW	92941	52980	408.74	395	422	SAMC798111
FW8	FW	86275	48301	409.68	395	422	SAMC798137
FW9	FW	82425	50606	407.51	395	422	SAMC798138
FW10	FW	81409	47539	411.59	260	422	SAMC798139
FW11	FW	72672	37028	408.33	257	422	SAMC798140
MF1	MF	77935	47389	408.57	395	421	SAMC798115
MF2	MF	82673	45365	410.01	307	422	SAMC798122
MF3	MF	93453	52888	406.79	257	422	SAMC798123
MF4	MF	92104	41266	401.99	259	422	SAMC798124
MF5	MF	90387	50096	411.24	395	422	SAMC798129
MM1	MM	82136	45335	408.02	395	422	SAMC798114
MM2	MM	104528	55091	410.22	395	422	SAMC798117
MM3	MM	92997	33970	408.92	258	422	SAMC798119
MM4	MM	96831	44585	411.18	395	422	SAMC798121
MM5	MM	94575	57840	407.83	395	421	SAMC798126
MM6	MM	88806	51696	408.94	395	422	SAMC798128
MW1	MW	98013	54728	407.02	395	422	SAMC798113
MW2	MW	86628	56998	403.80	283	422	SAMC798116
MW3	MW	97957	70420	405.41	260	422	SAMC798118
MW4	MW	90098	60973	406.60	260	423	SAMC798120
MW5	MW	96383	51194	405.50	258	422	SAMC798125
MW6	MW	90030	47425	413.33	395	422	SAMC798127
MW7	MW	95586	53061	408.23	258	422	SAMC798133
MW8	MW	71594	47456	408.50	395	422	SAMC798134
MW9	MW	89601	45778	406.39	258	422	SAMC798135
MW10	MW	90782	53788	406.60	257	422	SAMC798136
MW11	MW	89660	55005	407.86	260	422	SAMC798141
MW12	MW	93227	55424	406.95	281	422	SAMC798142
MW13	MW	73681	39467	410.10	395	422	SAMC798143

Table A2. The number of bacterial amplicon sequence variants (ASVs) at different taxonomic levels in each fecal sample.

Sample ID	Group	ASVs	Genus	Family	Class	Order	Phylum
FF1	FF	211	68	46	14	29	8
FF2	FF	189	78	52	15	34	10
FF3	FF	146	43	34	13	25	8
FF4	FF	197	84	52	15	35	10
FF5	FF	211	59	35	12	21	9
FF6	FF	173	61	41	14	29	8
FF7	FF	132	41	31	12	19	8
FM1	FM	213	71	43	11	24	7
FM2	FM	189	75	51	14	32	10
FM3	FM	179	52	36	12	23	7
FM4	FM	188	60	40	12	27	8
FM5	FM	162	49	38	12	23	7
FM6	FM	178	72	45	12	27	8
FM7	FM	197	80	49	14	31	10
FW1	FW	126	55	34	10	21	7
FW2	FW	179	50	37	12	24	8
FW3	FW	167	51	37	10	21	7
FW4	FW	191	59	37	12	25	8
FW5	FW	115	41	29	10	19	7

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FW6	FW	206	56	38	13	26	8
FW7	FW	145	42	28	10	19	7
FW8	FW	147	41	28	10	18	7
FW9	FW	173	56	40	11	23	6
FW10	FW	114	35	32	12	22	7
FW11	FW	171	48	31	12	21	9
MF1	MF	122	35	30	12	22	8
MF2	MF	150	43	32	13	21	8
MF3	MF	142	45	33	12	22	8
MF4	MF	187	53	34	12	22	7
MF5	MF	149	43	32	11	19	8
MM1	MM	197	58	38	11	22	8
MM2	MM	212	56	37	11	22	6
MM3	MM	214	60	39	13	25	9
MM4	MM	186	46	31	13	21	8
MM5	MM	114	34	33	11	23	6
MM6	MM	210	58	44	12	27	7
MW1	MW	204	54	33	13	23	8
MW2	MW	134	52	40	11	28	7
MW3	MW	128	59	44	14	28	9
MW4	MW	183	77	47	13	30	9
MW5	MW	204	50	33	11	20	7
MW6	MW	147	36	24	11	16	7
MW7	MW	190	48	29	12	19	8
MW8	MW	117	39	28	12	19	8
MW9	MW	172	53	35	14	23	9
MW10	MW	161	48	31	12	20	8
MW11	MW	152	58	40	14	27	9
MW12	MW	135	44	26	10	18	7
MW13	MW	173	50	35	12	22	7

Table A3. The relative abundance of unique bacterial taxon among different groups based on Kruskal-Wallis H test. The letters "f" and "g" indicate family and genus, respectively.

Taxonomy	*df*	*H*	*p*
f__Caulobacteraceae	5	11.76	0.04
f__Desulfovibrioria	5	13.85	0.02
f__Eggerthellaceae	5	17.08	0.004
f__Erysipelatoclostridiaceae	5	11.23	0.05
f__Marinifilaceae	5	13.23	0.02
f__Tannerellaceae	5	10.51	0.06
g__*Bacteroides*	5	17.77	0.003
g__*Clostridium_sensu_stricto_1*	5	14.60	0.01
g__*Desulfovibrio*	5	16.18	0.006
g__*Eggerthella*	5	15.56	0.008
g__*Odoribacter*	5	13.62	0.02

Table A4. The relative abundance of unique predicated functions among different groups based on Kruskal-Wallis H test.

Level name	*df*	*H*	*p*
Metabolism|Energy metabolism	5	15.66	0.008
Environmental Information Processing	5	13.22	0.02
Environmental Information Processing|Membrane transport	5	13.54	0.02
Metabolism|Biosynthesis of other secondary metabolites	5	12.35	0.03
Metabolism|Metabolism of cofactors and vitamins	5	12.03	0.03
ko00010	5	11.47	0.04
ko00051	5	11.26	0.05
ko00340	5	12.82	0.02
ko00473	5	11.90	0.04
ko00720	5	14.23	0.01
ko00790	5	12.88	0.02
ko01055	5	13.40	0.02

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