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Letter to the editor Open Access

Characterization of gut microbial alterations in cynomolgus macaques during growth and maturation

Aging is closely related to physiology and disease development in animals. Gut microbiota varies with lifecycle and exerts profound influences on the host. To investigate gut microbial alterations during growth and maturation, 41 female cynomolgus monkeys (*Macaca fascicularis*) ranging in age from 1 month to 15 years were divided into four groups (infant, young, adult, and middle-aged). 16S rRNA gene analyses revealed that gut microbial composition in the infant monkeys was distinct from that in the young, adult, and middle-aged monkeys. With increasing age, gut microbiota richness and diversity increased and then remained steady in the young, adult, and middle-aged monkeys. At the genus level, unidentified *Prevotellaceae* and *Bifidobacterium* were abundant in the infant monkeys, while *Streptococcus* and unidentified *Ruminococcaceae* were enriched in the young, adult, and middle-aged monkeys. Importantly, microbial function in the infant monkeys differed significantly from that in the other groups. This study improves our understanding of gut microbial alterations during growth and maturation in cynomolgus macaques and demonstrates that gut microbial composition and function differ considerably in infant monkeys compared to young, adult, and middle-aged groups.

Gradual decline in principal physiological systems is an important feature of aging. To eliminate the effects of external factors (e.g., lifestyle, diet, nutrition), animal models are widely used in the study of aging. As non-human primates (NHPs) share similar genetic, physiological, and behavioral traits with humans, they are regarded as ideal models for studying aging processes.

The gut microbiota is a microbial ecosystem consisting of bacteria, archaea, viruses, fungi, and protozoa. It contains more than 100 trillion microbial cells and forms a complex community in the mammalian gastrointestinal tract. Importantly, the gut microbiota maintains a mutualistic relationship with the host and exerts a profound influence on host health (Bajaj, 2019; Siegel et al., 2019; Tang et al., 2017).

The composition and function of the gut microbiota vary greatly at different life cycle phases (Lim et al., 2015). To explore age-related gut microbial alterations, 16S rRNA and shotgun metagenomic sequencing technologies have been applied in NHP and human studies. Previous research on human gut microbial alterations from newborns to centenarians (0–104 years) showed several important patterns and transition points in the compositional changes in gut microbiota (Odamaki et al., 2016). In NHPs, although many important findings have been reported, information on the gut microbiota during early life (preweaning) remains scarce. For example, in previous studies, the youngest groups of marmosets and cynomolgus macaques were two years old (Duan et al., 2019; Reveles et al., 2019) and the youngest groups of golden snub-nosed monkeys and rhesus macaques were one and three years old, respectively (Adriansjach et al., 2020; Yao et al., 2021). As maturation of the gut microbiota and immune system is mainly accomplished during infancy, it is important to investigate gut microbial characteristics during this period.

In the current study, 41 female cynomolgus monkeys were selected based on age and divided into four groups: i.e., group A, eight infant monkeys (1 to 3 months old); group B, 10 young monkeys (2 to 5 years old); group C, 14 adult monkeys (7 to 10 years old); group D, nine middle-aged monkeys (12 to 15 years old) (Figure 1A). Fecal samples were collected to perform 16S rRNA gene sequencing. As seen in the Venn diagram (Figure 1B), 1 187 operational taxonomic units (OTUs) were shared by all monkeys. As shown in Figure 1C, gut microbiota richness and community diversity were

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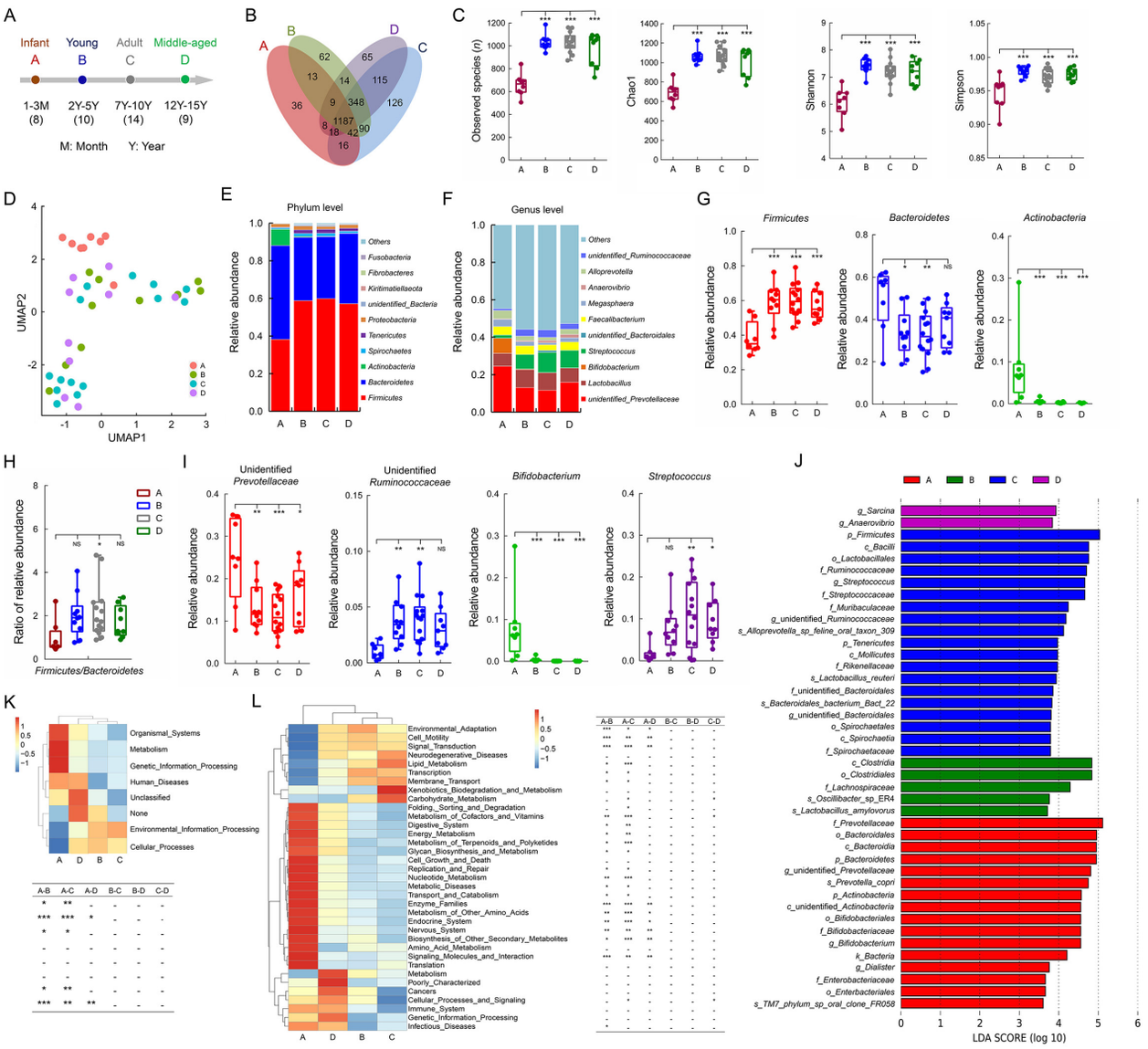

Figure 1 Characterization of age-related gut microbial alterations in cynomolgus macaques

A: Monkeys used in this study. M, month; Y, year. A, Infant monkey; B, Young monkey; C, Adult monkey; D, Middle-aged monkey. Number of monkeys is listed in brackets. B: Venn diagram of different OTUs. C: Richness and sample diversity. D: Uniform Manifold Approximation and Projection (UMAP) analysis comparing differences in gut microbiota information among four monkey groups. E, F: Relative abundances of OTUs assigned at phylum and genus levels, respectively. G: Relative abundances of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. H: *Firmicutes*/*Bacteroidetes* ratio. I: Relative abundances of unidentified *Prevotellaceae*, *Streptococcus*, unidentified *Ruminococcaceae*, and *Bifidobacterium*. J: LEfSe analysis of taxonomic differences among four groups. K, L: Heatmaps of functional differences in predicted genes in 16S rRNA gene sequencing related to KEGG pathways at level 1 and level 2, respectively. Data in C and G–I are presented as mean±standard error of the mean (*SEM*). Statistical significance of these data was analyzed by one-way analysis of variance (ANOVA) (***: $P<0.001$; **: $P<0.01$; *: $P<0.05$). Statistical significance between two groups in K and L was analyzed using *t*-test (***: $P<0.001$; **: $P<0.01$; *: $P<0.05$; –: $P>0.05$; NS: No significant difference).

markedly lower in the infant monkeys than in the young, adult, and middle-aged groups, indicating that more bacteria were colonized in the gastrointestinal tract of cynomolgus monkeys with age. Uniform Manifold Approximation and Projection (UMAP) analysis was used to examine the relationships between gut microbiota of the four groups. Consistent with α-diversity analysis, the gut microbiota in the infant monkeys differed from that in the other three groups (Figure 1D).

We next examined potential differences in bacterial taxa among the infant, young, adult, and middle-aged monkeys at the phylum, family, and genus levels (Figure 1E, F; Supplementary Figures S1–S3 and Tables S1–S3). At the phylum level, the relative abundance of *Firmicutes* was low in infant monkeys compared to that in young, adult, and middle-

aged monkeys; in contrast, *Bacteroidetes* and *Actinobacteria* were abundant in infant monkeys but decreased with age (Figure 1E, G). Furthermore, the *Firmicutes*/*Bacteroidetes* ratio was low in infant monkeys but increased in the other three groups, indicating that gut microbial composition and structure in cynomolgus macaques undergoes significant changes, especially from the infant to young stage (Figure 1H). At the family level, *Prevotellaceae* and *Bifidobacteriaceae* were enriched in infant monkeys but decreased in the other groups, while *Ruminococcaceae* and *Streptococcaceae* showed the reverse pattern (Supplementary Figure S2). At the genus level, unidentified *Prevotellaceae* and *Bifidobacterium* were abundant in the infant monkeys, whereas *Streptococcus* and unidentified *Ruminococcaceae* were enriched in the other groups (Figure 1I).

To identify distinct taxa among the four groups, linear discriminant analysis (LDA) effect size (LEfSe) (LDA>3.5, P<0.05) was used to analyze the 16S rRNA gene sequencing results. As shown in Figure 1J and Supplementary Figure S4, *Prevotellaceae*, *Bifidobacteriaceae*, and *Enterobacteriaceae* were enriched in the infant monkeys; *Lachnospiraceae* was abundant in the young monkeys; *Ruminococcaceae*, *Streptococcaceae*, *Muribaculaceae*, *Rikenellaceae*, and *Spirochaetaceae* were enriched in the adult monkeys; and *Sarcina* and *Anaerovibrio* were abundant in the middle-aged monkeys.

The functions of the gut microbial communities were analyzed using PICRUSt 2.0. Among the first-level Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, functions related to organismal systems, metabolism, and genetic information processing were enriched in the infant monkeys, while functions related to cellular processes and environmental information processing were abundant in the young, adult, and middle-aged monkeys (Figure 1K). For the second-level KEGG pathways, functions related to environmental adaptation, cell motility, signal transduction, lipid metabolism, transcription, and membrane transport were enriched in the young, adult and middle-aged monkeys, while functions related to metabolism of cofactors and vitamins, digestive system, energy metabolism, metabolism of terpenoids and polyketides, glycan biosynthesis and metabolism, replication and repair, nucleotide metabolism, metabolic diseases, transport and catabolism, enzyme families, metabolism of other amino acids, endocrine system, nervous system, biosynthesis of other secondary metabolites, and signaling molecules and interaction were enriched in the infant monkeys (Figure 1L). Moreover, functions related to carbohydrate metabolism was enriched in the young and adult monkeys, while functions related to infectious diseases were abundant in the infant and middle-aged groups (Figure 1L).

Comparing our results to those reported in humans (Odamaki et al., 2016), the NHP and human gut microbial community structures showed similar changes with increasing age: i.e., increase in relative abundance of *Firmicutes* and decrease in relative abundances of *Bacteroidetes* and *Actinobacteria* (Figure 1E, G). *Actinobacteria* was only abundant in the gut microbiota of infant-stage monkeys, in accordance with that found in the gut microbiota of humans at the preweaning stage. However, we noted several gut microbial differences between the NHPs and humans. At the preweaning stage, *Actinobacteria* represented the largest phylum in humans, whereas *Bacteroidetes* was the dominant phylum in NHPs. Furthermore, in humans, the relative abundance of *Proteobacteria* was high in the youngest (1 to 2 years old) and oldest groups (80 to 100 years old) (Odamaki et al., 2016), but showed no significant differences among the four NHP groups (Supplementary Figure S1). Moreover, *Actinobacteria* was still found in the gut microbiota of 40 to 49-year-old humans (relative abundance>10%), but was barely detected in the young, adult, and middle-aged cynomolgus macaques (relative abundance<0.5%). Regarding gut microbial function, lower xylose transporter abundance was reported in pre-weaned human infants, while all drug transporters were enriched in the infant/elderly humans. In NHPs, pathways related to membrane transport were enriched in the young, adult, and middle-aged monkeys, whereas pathways related to transport and catabolism were enriched in the infant group (Figure 1L).

In summary, we studied gut microbial alterations in cynomolgus macaques during growth and maturation. Results showed that gut microbiota in the infant cynomolgus macaques differed markedly from that in the other groups from the following three aspects: (1) gut microbiota species richness and community diversity were lower in the infant monkeys than in the young, adult, and middle-aged monkeys (Figure 1C); (2) relative abundances of specific bacterial taxa changed significantly from the infant to young stage, especially bacteria belonging to *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Figure 1G , I); (3) gut microbial function was distinct in the infant monkeys compared to the young and adult monkeys, but showed some similarities with the middle-aged monkeys (Figure 1K, L). These results illustrate the unique gut microbiota of infant monkeys and emphasize the necessity to characterize the microbial features of these monkeys. However, several limitations exist in this study. Firstly, constrained by the scarcity of old monkeys, monkeys exceeding 20 years old were not included in this study. Secondly, as our work was a cross-sectional study, causal inferences related to age-based gut microbial changes cannot be made. Thirdly, gut microbial changes were only derived from female cynomolgus macaques. However, as gut microbiota in rhesus macaques shows no obvious sex differences (Adriansjach et al., 2020), the results generated in this work may also be suitable for males.

DATA AVAILABILITY

The 16S rRNA gene sequencing data used in this study were deposited in the Sequence Read Archive of NCBI with Accession No. SRP339009.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y.P.Y., Y.L., P.J.Y., Q.M.L., C.S.G., and X.T.Z. performed the experiments. Q.S. and Y.P.Y. conceived and supervised the study, analyzed the results, and wrote the manuscript. All authors contributed to manuscript revision and read and approved the final version of the manuscript.

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