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Do Gut Microbiota Correlate with Stereotypic Behavior? First Answers from Malayan Sun Bears and Insights into Ex-situ Conservation

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Abstract

Gut microbiota affect host health and behavior throughout life but their relationship in endangered animals is poorly understood. Malayan sun bears are protected as an endangered wild species. Abnormal behavior such as stereotypies can be used as welfare indicators of animals in zoos. In this study, the abundance and diversity of fecal microbiota from six Malayan sun bears in Tianjin Zoo were assessed using 16S rRNA Illumina technology, and stereotypies were analyzed to identify behavioral patterns in the bears, separated into two age groups. Stereotypic behavior did not differ significantly between bears of different ages, but bacterial diversity in bears from the elderly group was significantly higher than that of the adult group. The gut microbiota of Malayan sun bears was dominated by Firmicutes (54.70%), Proteobacteria (41.90%), and Fusobacteria (1.80%). Findings from this study facilitate understanding of the relationship between gut microbiota and behavioral patterns, which could benefit the welfare of animals in captivity.

Introduction

Gut microbiota diversity and composition have been extensively researched in wild animals [1, 2]. Fundamental studies on the microbiome-gut-brain axis have revealed compelling effects of gut microbes on host health, central neurochemistry, and behavior [1, 3]. The Malayan sun bear (*Helarctos malayanus*) mainly inhabits the rainforests of South-east Asia and is the smallest bear species listed as 'vulnerable' by the International Union for Conservation of Nature (IUCN) [4, 5]. The wild population of Malayan sun bears has declined by more than 30% in recent years because of habitat loss and the pet/entertainment trade, and consequently,

there is an urgent need for ex-situ conservation of this species [6, 7]. Zoos can offer residence to these bears and the bears may become habituated to humans in living condition and diets. Captivity can provide a beneficial living environment but can also negatively impact animal welfare because captivity restricts the ability of many species to perform natural behaviors due to the difference in environment and resources provided compared with the wild habitat [8, 9].

Stereotypies, a common example of abnormal behaviors, are defined as repetitive patterns and are attributed to boredom or stress in captive animals, indicating a habitual coping mechanism established in response to poor welfare [10-12]. Stereotypic behavior has been associated with poor mental or physical health condition in Malayan sun bears [8, 12], and linked with poor living conditions in brown bears (*Ursus arctos yesoensis*) [13]. Captivity restricts many natural behaviors due to the difference in environment. However, polar bears (*Ursus maritimus*) in captivity exhibited reduce stereotypies and increased play when welfare conditions were improved [6, 14, 15]. Thus, it is important to investigate the possible causes of stereotypies because it may reflect a mechanism established in response to living conditions for Malayan sun bears in zoos.

Gut microbiota are instrumental in maintaining health since microbiota closely influence digestion, metabolism, immunity, and development of wild animals [16, 17]. The establishment of gut microbiota in an animal starts through contact with their mother and microbial composition changes during their growth [18]. The age of animals and habitat conditions contribute to the establishment of complex microorganisms, and gradually a stable intestinal microflora is formed through mutual adaptation and co-evolution with the host [18-20]. The gut microbiota is a complex ecosystem and maintain a dynamic balance and stability through interaction, which has been shown to be directly related to the health of non-human animals [2, 21]. In recent years, studies of model animals such as mice and domestic animals such as cows have covered a broad array of gut microbiota research [22, 23]. However, wild animals including some endangered species are the least-investigated animals because of their complex environments [24].

Studying gut microbiota in wild animals could offer opportunities to understand “real-life” microbiota and facilitate enhanced survival of animals in captivity. The gut microbiota of mammals has been studied in Andean bears [25], grizzly bears [26], polar bears (*Ursus maritimus*)[27], and giant pandas (*Ailuropoda melanoleuca*) [28, 29]. Such studies demonstrated that co-evolution exists between mammalian gut microorganisms and hosts, and that gut microbiota composition is closely related to the evolutionary course, feeding composition, and digestion of hosts [30, 31]. In comparison, there are relatively few studies on the gut microbiota of wild animals as well as their function on animal behavior, and most of these studies focused on the characteristics of natural microbiota [32]. Differences in gut microbes of wild animals were found when the living environment changed, with many natural microbes being lost following captivity [28, 33]. Thus, studying the gut microbiota and animal behavior might facilitate the provision of beneficial welfare conditions for captive rehabilitant Malayan sun bears [34].

High-throughput sequencing technology based on the 16S rRNA gene has been widely used for microbial community detection, and more and more gut microbiota from wild animals have been described and studied using this technology [35, 36]. This allows analysis of microbiota composition and can reveal interaction(s) with host behavior [35, 37]. In the current study, high-

throughput sequencing technology was employed to analyze the fecal microflora of Malayan sun bears of different ages, and stereotypical behaviors of the bears were assessed to identify behavioral patterns that were indicative of the welfare state of the bears in captivity. The purpose of the study was to explore the diversity composition of intestinal microflora of Malayan sun bears and identify, for the first time, relationship(s) between animal behavior and fecal microflora in captive Malayan sun bears. Insights gained from the behavior of these bears and their differing eds could be used to inform the management and husbandry of these animals. Research on intestinal flora of Malayan sun bears will provide more accurate and scientific data resources, and also lay a theoretical foundation for molecular biology research on the intestinal flora of *H. malayanus* and for reducing stereotypies in captive animals.

Results

Effect of age on animal behavior

The behavior of Malayan sun bears in Tianjin Zoo was defined in an ethogram shown in Table S1. To examine whether the presence of stereotypies was associated with age alterations, individual activity budgets were calculated and the difference in the stereotypical behavior grouped according to age was also analyzed, including mechanically repeat walking, licking genitals, and so on. Individual Malayan sun bears in Tianjin Zoo differed greatly in their budgets for active and stereotypic behavior type, but there was no significant difference between groups A and B (Fig. 1a).

Bear M6 (Stereotypical behavior ratio, 42.32%) performed more stereotypical behaviors than the other bears, and the main stereotypic behavior of M6 was pacing along the moat in the exhibition area. M2 (Stereotypical behavior ratio, 35.91%) and M4 (Stereotypical behavior ratio, 33.82%) displayed a larger variation in stereotypical behavior, while M1 (Stereotypical behavior ratio, 22.62%) and M5 (Stereotypical behavior ratio, 20.12%) showed more interest in interacting with visitors and had a lower incidence of stereotyped behavior. Although the incidence of stereotypical behavior varied widely from M1 to M6, there was no significant difference between the two age groups of bears (Fig. 1b).

Active behaviors were similar in all bears except M6. M5 (active behavior ratio,59.26%) exhibited slightly more active behavior than M1 (active behavior ratio,48.60%) and M4 (active behavior ratio,38.82%); however, there was no significant difference in the incidence of normal active behavior between M1 and M4. M3 (active behavior ratio,26.21%) was more inactive than M2 (active behavior ratio,32.11%), while M6 (active behavior ratio,0.62%), the oldest bear in the study, was the most inactive among all the bears. However, there was no significant difference of active behavior between two groups (Fig. 1c).

The average relative activity of captive Malayan sun bears was 0.34 ± 0.11, while the average incidence of stereotypical behavior was 0.30 ± 0.11. There were no significant differences among stereotypies and active behaviors in the two different age groups of bears.

Sequencing data analysis

Across samples from the six Malayan sun bears in the present study, a total of 420,131 raw sequencing reads were obtained after denoising by Illumina NovaSeq platform; raw reads per sample varied from 64,106 to 73,253. To establish whether the sequences covered all microbial species, the abundance curve of α-diversity, including Chao1, Shannon, and Simpson indices, was used to evaluate the accuracy of the analyses (Fig. 2). The curves tended to be flat when the number of sequences was greater than 4,000, indicating that increasing the number of sequencing reads would not yield additional operational taxonomic units (OTUs) and the current samples were large enough to identify the majority of OTUs (Fig. 2).

The value of α-diversity indices from Malayan sun bears are shown in Table S2. Bacterial community diversity and richness measured by OTUs (Chao1, Shannon, and Simpson indices) showed that the index of B group (elderly bears) was slightly higher than that of A group (adults). Although the sample size in the study was too small to test for significance, the difference in bacterial species diversity and richness tended to be greater for the elderly group (group B) compared with the adult group (group A) (Fig. S1). Average Good's coverage was 100% around all six samples, indicating that most of the bacterial diversity in the feces was obtained.

Bacterial community composition in Malayan sun bears

Bacterial community composition of the Malayan sun bears, based on 16S rRNA gene sequencing, was examined for potential differences according to age group of the bears. Bacterial composition of each fecal sample at different taxonomic levels is shown in Figs. 3, S2, and S3. Further examination of how these changes in phylum or genus composition affected the bacterial diversity as well as the richness of each age group was conducted.

At the phylum level, gut microbiota of Malayan sun bears from A group was dominated by Firmicutes (76.90%), Proteobacteria (22.41%), and Fusobacteria (0.60%). These three phyla accounted for 99.93% of all sequences across all the samples, while unclassified bacterial sequences only occupied 0.07% (Fig. 3a). The gut microbiota from B group was also dominated by Firmicutes (55.59%), Proteobacteria (42.81%), and Fusobacteria (1.53%) (Fig. 3a, Table 1), although the proportions differed to those of A group. Firmicutes was the most abundant phylum in both groups, but the relative abundance of this phylum was slightly lower in the elderly group (group B) compared with the adult group (group A). The second most common phylum in the two groups was the Proteobacteria, and the elderly group (group B) had a higher proportion of this phylum compared with the adult group (group A).

At the genus level, the most abundant genera in A group were *Streptococcus* (57.18%), *Escherichia-Shigella* (20.89%), *Lactobacillus* (10.67%), *Clostridium_sensu_stricto_1* (4.10%), and *Enterococcus* (2.50%). The most abundant genera in B group were *Escherichia-Shigella* (34.17%), *Streptococcus* (24.99%), *Clostridium_sensu_stricto_1* (13.48%), *Romboutsia* (3.54%), and *Klebsiella* (3.24%). Community heatmap analysis and mean relative abundances at the genus level of all six samples are displayed in Fig. 3b and Table 2. The most common genus in the adult group (group A) was *Streptococcus*, followed by *Escherichia-Shigella.* In contrast, the opposite trend was observed in the elderly group (group B), with *Escherichia-Shigella* as the most abundant genus, followed by *Streptococcus*. The Class_Circos and heatmap of the two groups are shown in Fig. 4.

β-Diversity analysis and community structures

To explore the relationship between fecal microbes and age, PCoA was used to visualize similarities or dissimilarities via a dendrogram using Bray–Curtis distances, and thus the difference in β-diversity of the six samples in the two groups could be evaluated (Fig. 5a). On the PCoA plot, there are six symbols, with each symbol representing one gut microbiota. Bacterial communities from the A group clustered tightly and were separated from those of the B group along principal coordinate axis 1 (PCoA1) with the largest amount of variation (74.87%) using weighted unifrac distance. This indicated that there was a clear grouping pattern that separated the two groups. Furthermore, the unique and shared bacterial taxa in all Malayan sun bears were analyzed. At the phylum level, 10 phyla were shared and there were only two unique phyla (Fig. S4a). At the genus level, 61 genera were shared as the core bacterial genus and the unique genera in each group were 33 (A group) and 56 (B group), respectively (Fig. S4b).

Given the suggested link between gut bacteria and host age in many animals, the similarities in gut microbiota from the two groups were analyzed. The top two abundant core phyla were Firmicutes and Proteobacteria, while the top two abundant core genera were *Streptococcus* and *Escherichia-Shigella* (Fig. 5b,5c). The relative abundance of known pathogenic species, focusing on Proteobacteria, was higher in group B than in group A, and there was a significant decrease in abundance of *Lactobacillus*, as commonly reported with aging. Bubble plot showing comparative OTU profiles could be seen in Fig.5d.

Conclusions

The intestinal microbiota of wild animals plays an important role in host metabolism, development, and behavior. Studies on the microbiome-gut-brain axis have revealed compelling effects of the microbiome on behavior and neurophysiology; however, how microbiota changes modulate host behavior and the microbial effects on each mode of behavior have yet to be elucidated [38-40]. In the current study, stereotypical behaviors were observed in all Malayan sun bears from Tianjin Zoo, which was similar to previous studies [5]. Age changes and gut microbiota construction in these bears were subsequently examined to determine the potential relationship between the factors and stereotypical behaviors [41].

The environment is quite different between wild habitats and zoos. Animals spend a lot of time searching for food with species-appropriate behaviors in the wild [6, 42], whereas the food is offered on a routine schedule, including the time to eat and the amount, when animals are kept in zoos [43, 44]. Stereotypical behaviors can occupy more than 50% of the time of a captive Malayan sun bear living in a minimal undersized habitat [8], thus the current study assumed this behavior was associated with poor physical health. All six Malayan sun bears in the current study exhibited stereotypical behaviors. Although the incidence of this behavior varies greatly between individuals, the overall prevalence of stereotypies was relatively low suggesting that the Tianjin Zoo habitat provided a relatively good living environment compared with other captive environments.

Stereotypies can be reduced using enrichment programs such as providing novel toys and

foraging opportunities, but other factors including seasonal changes, visitor density, and the relationship with the construction of gut microbes must be explored as possible causes of stereotypical behaviors in captive Malayan sun bears [45]. In a previous study, stereotypical behaviors increased when visitors were present at a relative high level. Seasonal changes, which are related to mating and forage-seeking behaviors in the wild, may affect stereotypical behaviors in captive polar bears and black bears [45].

This study analyzed, for the first time, stereotypical behaviors of Malayan sun bears in relation to the composition of gut microbiota. The gut microbiota, associated with many diseases including allergies, diabetes, and obesity, could affect each Malayan sun bear of different ages in a unique manner. In healthy individuals, the gut bacterial community is mainly dominated by the phyla Firmicutes, and this result was similar in the two groups of Malayan sun bears. Through analysis of the sequencing results, Firmicutes and Proteobacteria were revealed as the dominant phyla in the feces of Malayan sun bears. Thus, the microbial community structure in fecal samples of Malayan sun bears is essentially the same as that of many other mammalian species, including giant panda, polar bear (*Ursus maritimus*), and black bear (*Ursus thibetanus*).

The main bacterial phyla in feces of giant pandas were Firmicutes and Proteobacteria. The intestinal flora of the black bear (*Ursus thibetanus*) was identified into 12 phyla including Firmicutes, Proteobacteria, and Fusobacteria. The construction was similar at the phyla level in Malayan sun bears, but at the genus level, the fecal flora of Malayan sun bears differed from those of giant panda and black bear. Fecal samples of Malayan sun bears had a high content of *Streptococcus*, followed by *Escherichia coli/Shigella*, and *Clostridium* at the genus level, while the dominant bacteria in the fecal flora of giant panda were *Clostridium* and *Escherichia coli/Shigella*. The dominant bacteria in the intestinal flora of polar bear (*Ursus maritimus*) and black bear are *Enterococcus*, and *Clostridium*. Reasons for the differences between these findings and the gut microbiota of Malayan sun bears were further analyzed. Although giant panda and Malayan sun bears both belong to the order Carnivora, their dietary habits are different. Giant pandas predominantly eat bamboo, while Malayan sun bears are omnivores. Polar bears, black bears, and Malayan sun bears also have different food preferences. Species differences may therefore be the main reason for the differences in intestinal flora among animals in the same family. In addition, the intestinal flora of wild animals is affected by many factors, such as food, individual body, age, and so on. By analyzing the intestinal flora of Malayan sun bears of different ages, it was revealed that the diversity in adult Malayan sun bears was the highest. Firmicutes occupied 76.90% in the adult group (A group), but only occupied 55.59% in the elderly group (B group). Members of the Proteobacteria, containing many pathogens, were more abundant in the elderly group (42.81%) than in the adult group (22.41%). Meanwhile, the main genera in the adult group were *Streptococcus* (57.18%) and *Escherichia coli* (20.89%). The main genera in the elderly group were *Escherichia coli*/*Shigella* (34.17%) and *Streptococcus* (24.99%). Generally, an increase in members of the Proteobacteria and lower overall community diversity were observed in the elderly group, indicating that the physical health of the elderly group was not as good as that of the younger group.

In this study, the dominant phyla in fecal samples of six Malayan sun bears were identical, but the content of the same phyla in fecal samples of different individuals was different. There were also differences in the number, species, and abundance of the microflora at the class,

order, family, and genus levels. The diversity of the fecal microflora of each Malaya sun bear was also different, indicating that there were individual differences and individual specificity in the fecal microflora of Malayan sun bears. The composition of mammalian intestinal flora changes with different hosts. Since the six sampled Malayan sun bears share the same diet and feeding environment, age is hypothesized to be the main factor responsible for the results of this study, in addition to individual animal life.

To ascertain whether the gut microbiota can affect behavior, the correlation between individual behavior and microbial ratio was explored (Table 3). Analysis of the relationship between the top five phyla/genera and the two behaviors revealed that *Streptococcus* and *Escherichia coli/Shigella* were negatively correlated with active behavior and positively correlated with stereotypical behavior. In contrast, *Clostridium* and *Lactobacillus* were positively correlated with active behavior and negatively correlated with stereotypical behavior. These results suggested that there were significant differences in intestinal flora between different ages, and that *Clostridium* and *Lactobacillus* may contribute to the expression of active behavior of Malayan sun bears. Furthermore, the relative abundance of known pathogenic bacterial species (mainly species of the genus *Proteus*) in the elderly group was higher than that in the adult group, and the abundance of *Lactobacillus* decreased significantly with increasing age. Therefore, the physical health of the older group was not as good as that of the younger group, and stereotyped behaviors occurred more frequently.

In summary, this study characterized the gut microbiota of Malayan sun bears using 16S rRNA gene sequencing analysis. The captive diet, feeding environment, and behavior pattern all directly affect the intestinal flora structure of these animals, but exactly how these factors influence the structure of flora composition remains a mystery. The incidence of stereotypic behavior in the elderly group (B group) was higher than that in the adult group (A group). There was no significant difference in stereotyped behavior according to age, but bacterial diversity was significantly higher in the older group than in the adult group. Firmicutes and Proteobacteria were the dominant flora in both groups, among which *Streptococcus*, *Escherichia coli/Shigella*, and *Clostridium* were the dominant genera. With the increase of age, the abundance of *Streptococcus* in the intestinal tract of Malayan sun bears gradually decreased, while the abundance of *Escherichia coli/Shigella* and *Clostridium* increased. This study revealed the intestinal flora of Malay bears and preliminarily explored the relationship between intestinal flora and behavioral patterns, which may have applications in helping to protect this endangered species.

Methods

Ethics information

All work including the sample collection and behavior observations complied with the current laws of China and followed applicable international and national guidelines. Fecal sample collections were conducted under permit without direct contact with the Malayan sun bears. All experimental procedures carried out were approved by the Forestry Bureau of Tianjin Province of China (approval number: [2020]17).

Subjects and behavior analysis

Behavioral observations were recorded from six individual Malayan sun bears at Tianjin Zoo, China. Although there were many sun bears in the Zoo, only those that could be easily identified or monitored by the observer were analyzed in this study. Samples and observations were collected from one male and five female bears ranging from 5 to 30 years old. The age, sex, and other information of the six Malayan Sun Bears is shown in Table S3. The bears were divided into two groups according to their ages: A group (adult, 3<age<15) and B group (elderly, age>15).

Each bear was observed for two sessions including each morning (between 0800 and 1100) and each afternoon (between 1300 and 1600), and data were typically collected 5 days a week, which allowed for behavioral differences arising from the time of day. Bears were typically on exhibit together in Tianjin Zoo, and their behaviors were divided into two categories: active behavior included all activities except sleeping, while stereotypical behavior mainly comprised mechanical repetitive behavior. All behavioral data were analyzed using SPSS 26.0 statistical software (IBM, Chicago, IL, USA). The two types of behaviors were analyzed by Mann–Whitney U test [46].

Collection and DNA extraction of fecal samples and construction of libraries

Fecal samples of Malayan sun bears were collected at Tianjin Zoo immediately after natural defecation and were transferred into 2-mL sterile cryopreservation tubes and stored at -80°C in the laboratory as soon as possible.

DNA was extracted from the fecal samples using QiAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's instructions. Total DNA was eluted in 50 µL ddH_2O and stored at -80°C until further use. DNA concentration was measured with an ultraviolet spectrophotometer, and all six samples were suitable for use in the construction of amplicon libraries. The V3-V4 region of the 16S rRNA gene was amplified by PCR based on methods in the literature and using the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') [47, 48]. The resulting amplicons were prepared for sequencing, and libraries were assessed with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). Libraries were sequenced by LC-Bio Technology Co., Ltd (Hang Zhou, Zhejiang Province, China) using a NovaSeq PE250 platform with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Bioinformatics, sequencing, and statistical analyses

All samples were sequenced and paired-end reads were assigned. These reads were truncated by removing the barcode and merged using FLASH [49]. Quality filtering on the raw

reads was performed under specific conditions to obtain high-quality clean tags according to the fqtrim (v0.94). Chimeric sequences were filtered using Vsearch software (v2.3.4). Alpha diversity and a species accumulation curve were calculated to show the complexity of gut microbiota species. The feature abundance of each sample was normalized by SILVA classifier using relative abundance. Alpha diversity, including Chao1, observed species, Good's coverage, Shannon, and Simpson indices, were calculated by QIIME2 microbiome bioinformatics platform (https://qiime2.org) to analyze the complexity of species diversity.

β-Diversity analysis was performed and principal coordinate analyses (PCoA) were determined to investigate structural variation in microbial communities using unweighted and weighted UniFrac distance metrics [50]. Sequence blast was used for alignment, and the feature sequences were annotated with the SILVA database. Taxa abundances at the phylum, class, order, family, and genus levels of all the fecal samples were statistically compared using the R package (v3.5.2) [51]. SPSS Statistics 21.0 (IBM) software was used to analyze the data and one-way analysis of variance (ANOVA) was used to evaluate the differences. $P<0.05$ was considered statistically significant.

References

1. Tilg, H. & Kaser, A. Gut microbiome, obesity, and metabolic dysfunction. *J. Clin. Invest.* **121** (6), 2126-32 (2011).
2. Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. & Gordon, J. I. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* **6** (10), 776-788 (2008).
3. Maynard, C. L., Elson, C. O., Hatton, R. D. & Weaver, C. T. Reciprocal interactions of the intestinal microbiota and immune system. Nature **489** (7415), 231-241 (2012).
4. Forbes, G., Crudge, B., Lewis, K., Officer, K. & Descovich, K. An observational study of the behaviour of captive rehabilitant sun bears (*Helarctos malayanus*). *J. Appl. Anim. Welf. Sci.* **24** (2), 159-172 (2021).
5. Izzat-Husna, M., Mansor, M. S., Nabilah, N., Abidin, K. Z., Kamarudin, Z., Topani, R. et al. Behavior patterns of captive Malayan sun bears (*Helarctos malayanus*) at a rehabilitation center in Peninsular Malaysia. *J. Vet. Behav.* **43**, 39-45 (2021).
6. Fernandez, E. J. Appetitive search behaviors and stereotypies in polar bears (*Ursus maritimus*). *Behav. Processes* **182**, 104299 (2021).
7. Chien, Y. C., Lien, C. Y., Guo, J. C., Chin, S. C., Chang, Y. P. & Liu, C. H. Meningothelial meningioma in a Malayan sun bear (*Helarctos malayanus*). *J. Vet. Diagn. Invest.* **25** (5), 636-40 (2013).
8. Tan, H. M., Ong, S. M., Langat, G., Bahaman, A. R., Sharma, R. S. & Sumita, S. The influence of enclosure *design on diurnal activity and stereotypic behaviour in captive Malayan Sun bears (Helarctos* malayanus). *Res. Vet. Sci.* 94 (2), 228-39 (2013).
9. Nazeri, M., Jusoff, K., Madani, N., Mahmud, A. R., Bahman, A. R. & Kumar, L. Predictive modeling and mapping of Malayan Sun Bear (*Helarctos malayanus*) distribution using maximum entropy. *PLoS One* **7** (10), e48104 (2012).
10. Jeong, D. H., Yang, J. J. & Yeon, S. C., Fluoxetine therapy to decrease stereotypic behavior in the Asiatic black bear (*Ursus thibetanus*). *J. Zoo. Wildl. Med.* **50** (3), 718-722 (2019).

11. Miller, L. J., Kuczaj, S. & Herzing, D. Stereotypic behavior in wild marine carnivores? *Zoo. Biol.* **30** (4), 365-70 (2011).
12. Clubb, R. & Mason, G. Animal welfare: captivity effects on wide-ranging carnivores. *Nature* **425** (6957), 473-4 (2003).
13. Ishikawa, A., Sakamoto, H., Katagiri, S. & Takahashi, Y. Changes in sexual behavior and fecal steroid hormone concentrations during the breeding season in female Hokkaido brown bears (*Ursus arctos yesoensis*) under captive condition. *J. Vet. Med. Sci.* **65** (1), 99-102 (2003).
14. Carlstead, K. & Seidensticker, J. Seasonal variation in stereotypic pacing in an American black bear *Ursus americanus*. *Behav. Processes* **25** (2-3), 155-61 (1991).
15. Cless, I. T. & Lukas, K. E. Variables affecting the manifestation of and intensity of pacing behavior: A preliminary case study in zoo-housed polar bears. *Zoo. Biol.* **36** (5), 307-315 (2017).
16. Pascoe, E. L., Hauffe, H. C., Marchesi, J. R. & Perkins, S. E. Network analysis of gut microbiota literature: an overview of the research landscape in non-human animal studies. *ISME J*. **11** (12), 2644-2651 (2017).
17. Wu, Q., Wang, X., Ding, Y., Hu, Y., Nie, Y., Wei, W. et al. Seasonal variation in nutrient utilization shapes gut microbiome structure and function in wild giant pandas. *Proc. Biol. Sci.* **284** 1862 (2017).
18. Round, J. L. & Mazmanian, S. K. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* **9** (5), 313-23 (2009).
19. Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. Human gut microbiome viewed across age and geography. *Nature* **486** (7402), 222-227 (2012).
20. Sommer, F., Stahlman, M., Ilkayeva, O., Arnemo, J. M., Kindberg, J., Josefsson, J. et al. The gut microbiota modulates energy metabolism in the hibernating brown bear *Ursus arctos*. *Cell Rep.* **14** (7), 1655-1661 (2016).
21. Molina-Torres, G., Rodriguez-Arrastia, M., Roman, P., Sanchez-Labraca, N. & Cardona, D. Stress and the gut microbiota-brain axis. *Behav. Pharmacol.* **30** (2 and 3-Spec Issue), 187-200 (2019).
22. Hooper, L. V. & Gordon, J. I. Commensal host-bacterial relationships in the gut. *Science* **292** (5519), 1115-1118 (2001).
23. Qin, W., Song, P., Lin, G., Huang, Y., Wang, L., Zhou, X. et al. Gut microbiota plasticity influences the adaptability of wild and domestic animals in co-inhabited areas. *Front. Microbiol.* **11**, 125 (2020).
24. Viney, M. The gut microbiota of wild rodents: Challenges and opportunities. *Lab. Anim.* **53** (3), 252-258 (2019).
25. Borbon-Garcia, A., Reyes, A., Vives-Florez, M. & Caballero, S. Captivity shapes the gut microbiota of Andean bears: Insights into health surveillance. *Front. Microbiol.* **8**, 1316 (2017).
26. Schwab, C., Cristescu, B., Boyce, M. S., Stenhouse, G. B. & Ganzle, M., Bacterial populations and metabolites in the feces of free roaming and captive grizzly bears. *Can. J. Microbiol.* **55** (12), 1335-46 (2009).
27. Glad, T., Bernhardsen, P., Nielsen, K. M., Brusetti, L., Andersen, M., Aars, J. et al. Bacterial diversity in faeces from polar bear (*Ursus maritimus*) in Arctic Svalbard. *BMC Microbiol.* **10**, 10 (2010).
28. Guo, W., Mishra, S., Wang, C., Zhang, H., Ning, R., Kong, F. et al. Comparative study of gut microbiota in wild and captive giant pandas (*Ailuropoda melanoleuca*). *Genes (Basel)* **10** (10) (2019).
29. Zhang, W., Yang, S., Shan, T., Hou, R., Liu, Z., Li, W. et al. Virome comparisons in wild-diseased and healthy captive giant pandas. *Microbiome* **5** (1), 90 (2017).

30. Yang, Y. Z., Deng, Y. & Cao, L. Characterising the interspecific variations and convergence of gut microbiota in Anseriformes herbivores at wintering areas. *Sci. Rep.* **6**, 32655 (2016).
31. Xue, Z., Zhang, W., Wang, L., Hou, R., Zhang, M., Fei, L. et al. The bamboo-eating giant panda harbors a carnivore-like gut microbiota, with excessive seasonal variations. *mBio* **6** (3), e00022-15 (2015).
32. Lavrinienko, A., Tukalenko, E., Kesaniemi, J., Kivisaari, K., Masiuk, S., Boratynski, Z., et al. Applying the Anna Karenina principle for wild animal gut microbiota: Temporal stability of the bank vole gut microbiota in a disturbed environment. *J. Anim. Ecol.* **89** (11), 2617-2630 (2020).
33. Comizzoli, P. & Power, M. Reproductive microbiomes in wild animal species: A new dimension in conservation biology. *Adv. Exp. Med. Biol.* **1200**, 225-240 (2019).
34. Karl, J. P., Hatch, A. M., Arcidiacono, S. M., Pearce, S. C., Pantoja-Feliciano, I. G., Doherty, L. A. et al. Effects of psychological, environmental and physical stressors on the gut microbiota. *Front. Microbiol.* **9**, 2013 (2018).
35. Manichanh, C., Chapple, C. E., Frangeul, L., Gloux, K., Guigo, R. & Dore, J., A comparison of random sequence reads versus 16S rDNA sequences for estimating the biodiversity of a metagenomic library. *Nucleic Acids Res.* **36** (16), 5180-8 (2008).
36. Schriefer, A. E., Cliften, P. F., Hibberd, M. C., Sawyer, C., Brown-Kennerly, V., Burcea, L., et al. A multi-amplicon 16S rRNA sequencing and analysis method for improved taxonomic profiling of bacterial communities. *J. Microbiol. Methods* **154**, 6-13 (2018).
37. Shakya, M., Quince, C., Campbell, J. H., Yang, Z. K., Schadt, C. W. & Podar, M. Comparative metagenomic and rRNA microbial diversity characterization using archaeal and bacterial synthetic communities. *Environ. Microbiol.* **15** (6), 1882-99 (2013).
38. Ezenwa, V. O., Archie, E. A., Craft, M. E., Hawley, D. M., Martin, L. B., Moore, J. et al. Host behaviour-parasite feedback: an essential link between animal behaviour and disease ecology. *Proc. Biol. Sci.* **283**, 1828 (2016).
39. Ghaisas, S., Maher, J. & Kanthasamy, A., Gut microbiome in health and disease: Linking the microbiome-gut-brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases. *Pharmacol. Ther.* **158**, 52-62 (2016).
40. Dinan, T. G. & Cryan, J. F., The microbiome-gut-brain axis in health and disease. *Gastroenterol. Clin. North Am.* **46** (1), 77-89 (2017).
41. Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. & Xavier, J. B. Microbiology. Animal behavior and the microbiome. *Science* **338** (6104), 198-199 (2012).
42. Fraser, D. Biology of animal stress: implications for animal well-being. *J. Appl. Anim. Welf. Sci.* **2** (2), 157-159 (1999).
43. Soriano, A. I., Vinyoles, D. & Mate, C., Long-term macroevaluation of environmental enrichment in three brown bears (*Ursus arctos*) at Barcelona zoo. *J. Appl. Anim. Welf. Sci.* **19** (1), 49-61 (2016).
44. Carlstead, K. A comparative approach to the study of keeper-animal relationships in the zoo. *Zoo. Biol.* **28** (6), 589-608 (2009).
45. Kelly, K. R., Harrison, M. L., Size, D. D. & MacDonald, S. E. Individual effects of seasonal changes, visitor density, and concurrent bear behavior on stereotypical behaviors in captive polar bears (*Ursus maritimus*). *J. Appl. Anim. Welf. Sci.* **18** (1), 17-31 (2015).
46. Altmann, J., Observational study of behavior: sampling methods. *Behaviour* **49** (3), 227-67 (1974).

47. Fuks, G., Elgart, M., Amir, A., Zeisel, A., Turnbaugh, P. J., Soen, Y. et al. Combining 16S rRNA gene variable regions enables high-resolution microbial community profiling. *Microbiome* **6** (1), 17 (2018).
48. Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7** (5), 335-6 (2010).
49. Edgar, R. C., UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **10** (10), 996-8 (2013).
50. Lozupone, C. & Knight, R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **71** (12), 8228-35 (2005).
51. Luo, C., Rodriguez, R. L. & Konstantinidis, K. T., A user's guide to quantitative and comparative analysis of metagenomic datasets. *Methods Enzymol*. **531**, 525-47 (2013).

Data Availability Statement

The raw 16S rRNA gene sequence data for the six Malayan sun bears have been submitted to the National Center for Biotechnology Information (NCBI) database (BioProject ID: PRJNA735515). The SRA accession numbers are SRR14748196-SRR14748201.

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Author contributions

N.W. and H.Y. conceived the project. X.G. developed the methods. S.Z. and Y.W. performed the research. H.W., Q.Z., and D.Z. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Figures

Figure Legends

Figure 1. Behavioral data analysis of animals. **(a)** Behavioral data statistics for each individual Malayan sun bear (including active behavior and stereotypies). **(b)** Differences in active behavior between the two groups of bears. **(c)** Differences in stereotypies between the two groups of bears.

Figure 2. Curves of alpha-diversity analysis. Curves are based on **(a)** Chao1, **(b)** observed-outs, **(c)** Shannon, and **(d)** Simpson indices. The x-coordinate is the number of sequences and each curve in the graph represents a sample, which is labeled with a different color.

Figure 3. Microbial structure of all fecal samples from Malayan sun bears at phylum and genus levels. Bar-plots show the abundance and distribution of **(a)** the 18 most abundant phyla and **(b)** the 30 most abundant genera.

Figure 4. Circlize diagram and community heatmap of gut microbiota. **(a)** Circlize diagram of A group and B group showing the correlation of sample species abundance. **(b)** Community heatmap analysis of A group and B group at genus level.

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Tables:

Table 1 Mean relative abundance of top five abundant phyla (%) in the Malayan sun bears

Sample group	Phylum (mean relative abundance, %)
A	Firmicutes (76.90)
	Proteobacteria (22.41)
	Fusobacteria (0.60)
	Actinobacteria (0.05)
	Bacteroidetes (0.01)
B	Firmicutes (55.59)
	Proteobacteria (42.81)
	Fusobacteria (1.53)
	Actinobacteria (0.04)
	Cyanobacteria (0.01)

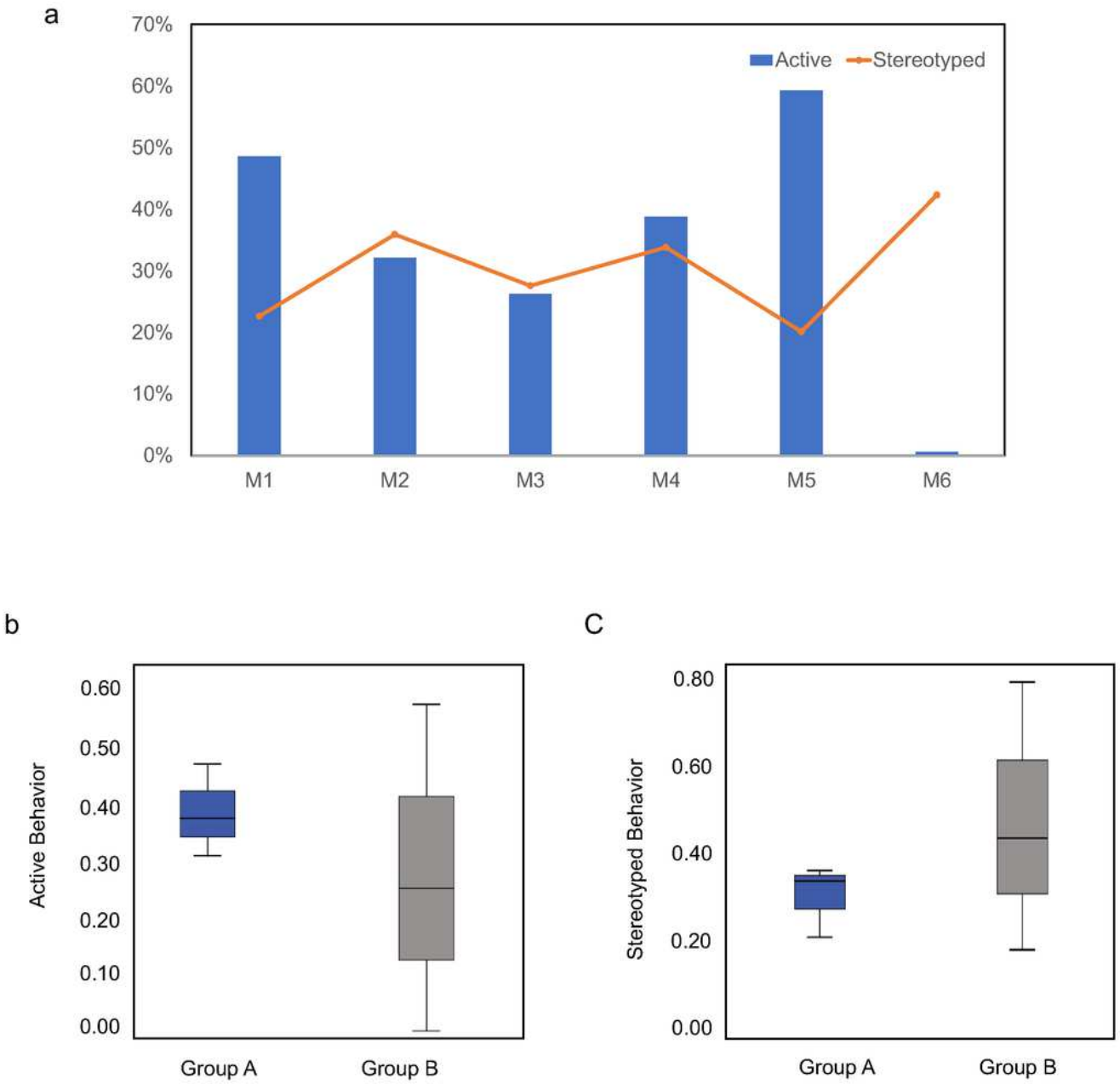
Table 2 Mean relative abundance of top five abundant genera (%) in Malayan sun bears

Sample group	Genus (mean relative abundance, %)
A	*Streptococcus* (57.18)
	Escherichia-Shigella (20.89)
	Lactobacillus (10.67)
	Clostridium_sensu_stricto_1 (4.10)
	Enterococcus (2.50)
B	*Escherichia-Shigella* (34.17)
	Streptococcus (24.99)
	Clostridium_sensu_stricto_1 (13.48)
	Romboutsia (3.54)
	Klebsiella (3.24)

Table 3 Correlation analysis between intestinal flora and animal behaviors (active behavior and stereotyped behavior)

Taxonomic classification level	Bacteria	Active behavior		Stereotyped behavior	
		r	p	r	p
Phylum	Firmicutes	-0.265	0.612	0.116	0.827
	Proteobacteria	0.246	0.639	-0.126	0.811
	Fusobacteria	0.267	0.609	0.115	0.827
	Actinobacteria	0.734	0.096	0.076	0.881
	Bacteroidetes	-0.405	0.426	0.765	0.076
Genus	*Streptococcus*	0.382	0.455	-0.298	0.566
	Escherichia-Shigella	-0.084	0.874	0.256	0.624
	Clostridium_sensu_stricto_1	-0.613	0.196	0.523	0.287
	Lactobacillus	0.320	0.526	-0.293	0.573
	Cetobacterium	0.267	0.609	-0.201	0.703

Figures



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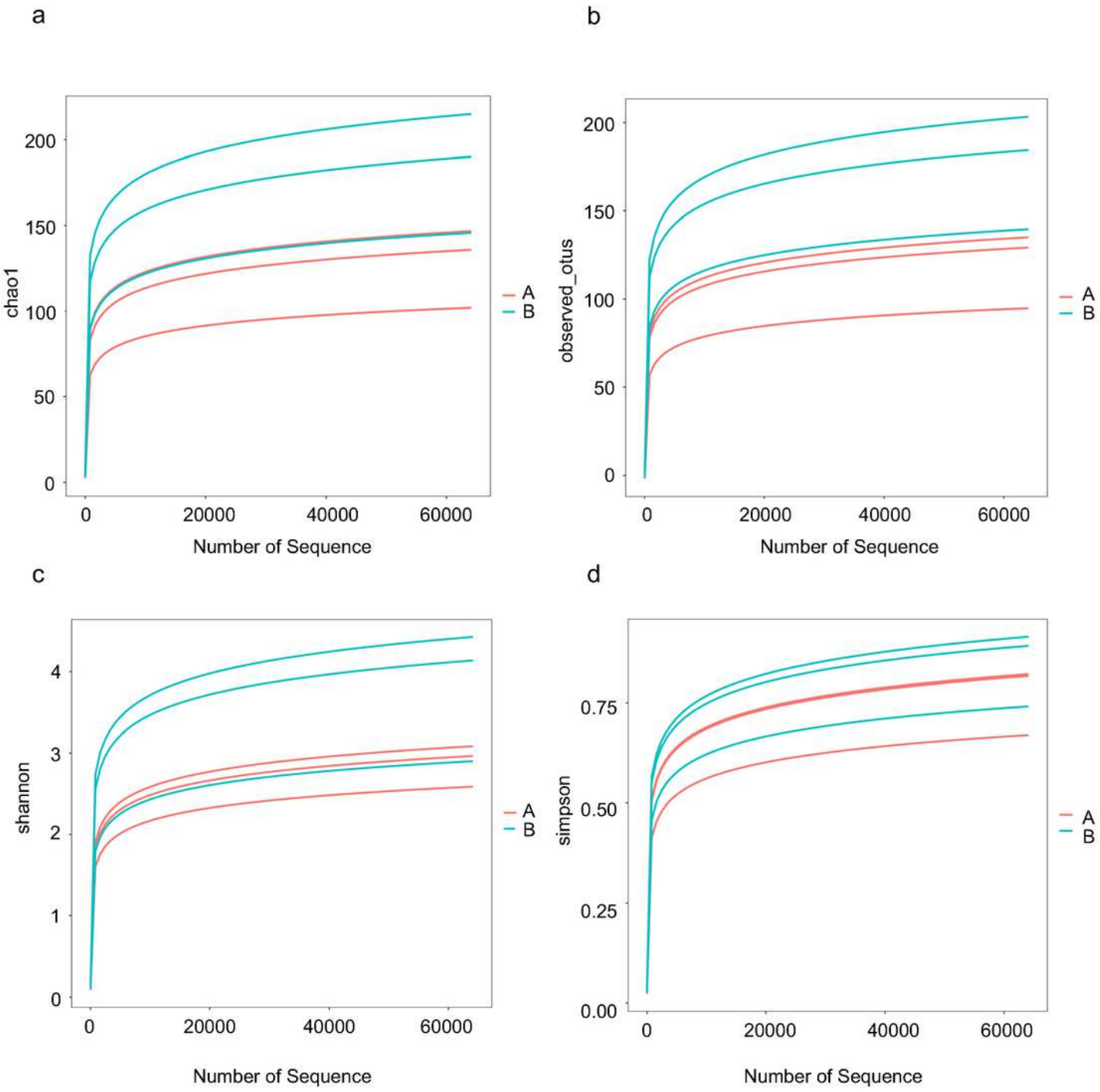

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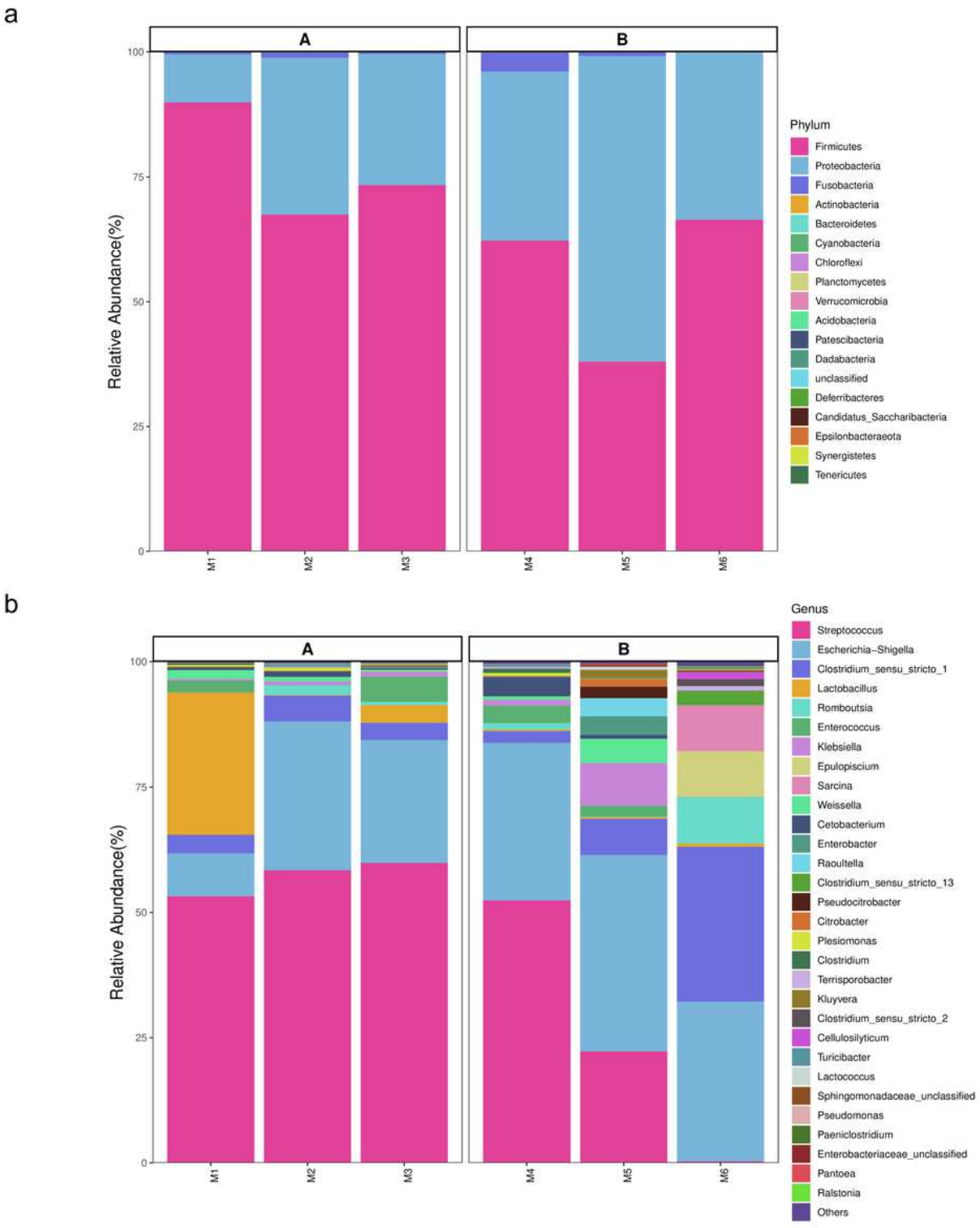

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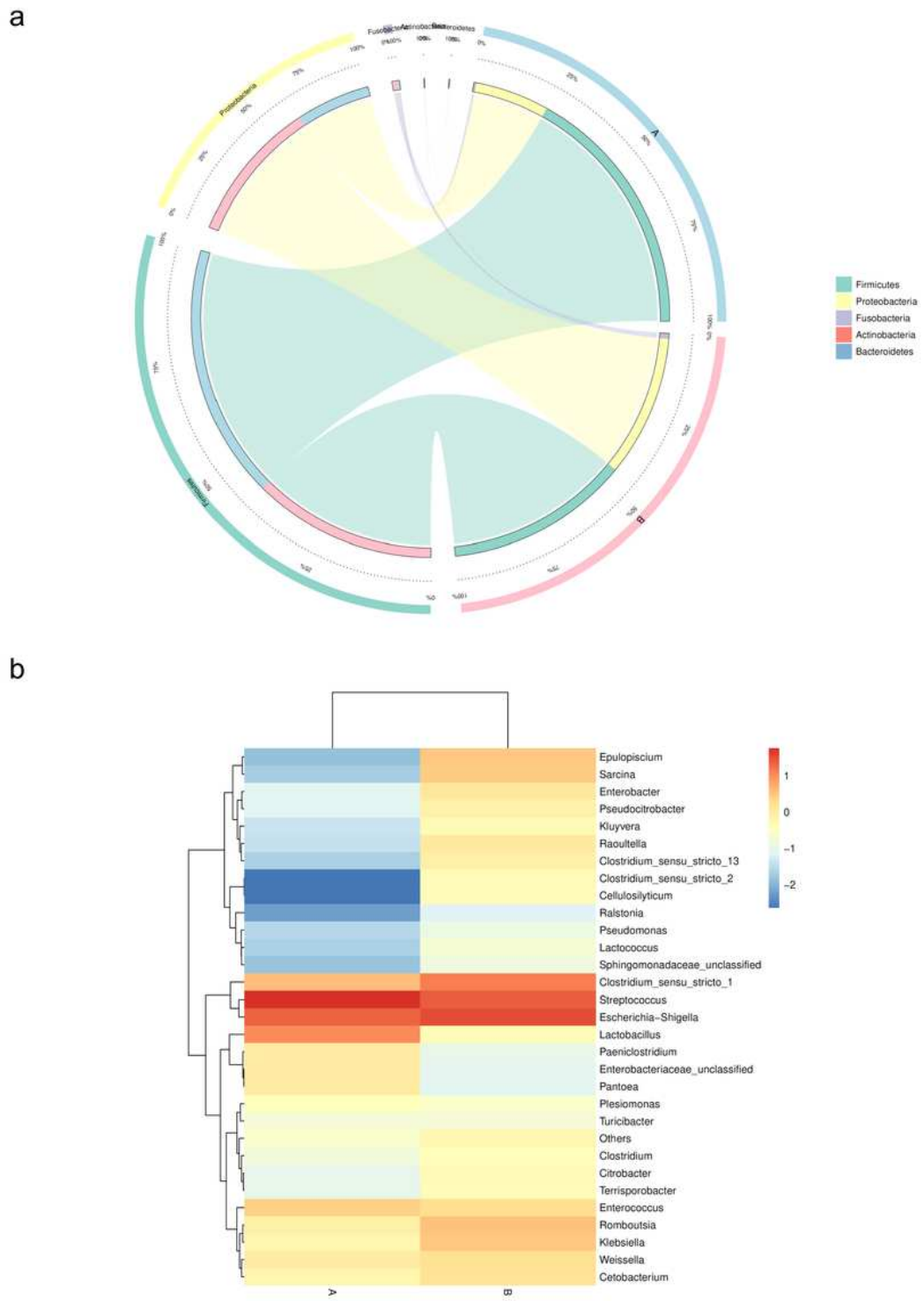

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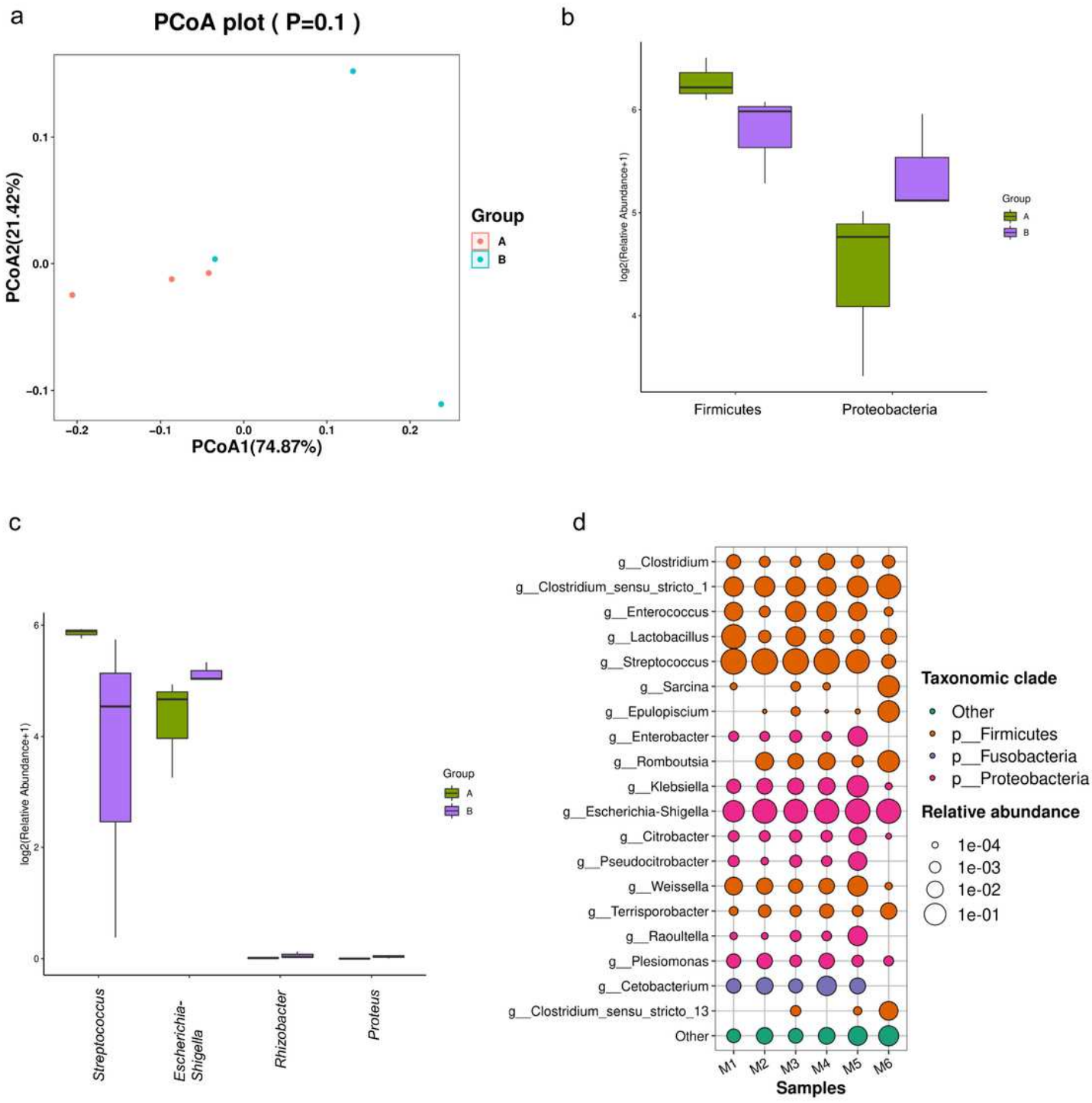

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Supplementary Files

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