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Research

Assessment of Gut Microbiome Variations in The Mother and Twin Infant of Captive White-Handed Gibbons (*Hylobates lar*) Reveals The Presence of Beneficial and Nonpathogenic Bacteria

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ABSTRACT

The white-handed gibbon (*Hylobates lar*) is one of the three Hylobatidae species found in the Peninsular Malaysia region. Studies on primate gut microbiota have yet to be conducted in Malaysian Hylobatidae, but previous studies have covered Cercopithecidae. Knowledge of the gut microbiota of endangered captive Malaysian Hylobatidae primates is important for health assessment and conservation management. Thus, the main objective of this study was to determine the gut microbiota profile of captive white-handed gibbons using the metabarcode 16S rRNA gene. Two *H. lar* fecal samples collected from the mother and twin infant at Zoo Melaka were used for DNA extraction. Next-generation sequencing (NGS) of the 16S rRNA gene was performed. The NGS data were analyzed on the basis of amplicon sequence variation. The gut microbiota of *H. lar* was dominated by phylum Firmicutes and Bacteroidota because of their important roles in fermentation and nutrient assimilation from plant-based food sources. Predominance of genus *Prevotella* in the gut microbiota of *H. lar* indicates that the captivity condition causes the gut microbiota of gibbons to resemble that of humans and associates with fiber-rich diets. Variation in the bacterial community profile between the mother and the infant indicated differences in dietary adaptations and physiological state. This study can be used as an indicator of gibbon health levels through gut microbiome screening and can be used when considering Malaysian primate health welfare and captive management.

Key words: Hylobatidae, next-generation sequencing, gut microbiome, small ape, white-handed gibbon

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INTRODUCTION

Animal gut microbiota refers to microorganisms found in the gastrointestinal system of a host animal. These symbiotic microbes are essential for animal survival and physiological processes (Borbón-García *et al*., 2017; Huan *et al*., 2020). The gut microbiota composition of a given host animal is determined by environmental factors (Barelli *et al*., 2015; Borbón-García *et al*., 2017), the host animal's nutrition (Zhu *et al*., 2018), and habitat quality, whether in the wild (Moeller *et al*., 2014) or in captivity (Hale *et al*., 2018). Recently, studies on gut microbiota have gained increasing attention for endangered species conservation (Li *et al*., 2022; Khairulmunir *et al*., 2023). Because non-human primates have close genetic and physiological relationships with humans, the study of gut microbiota in non-human primates is important (Li *et al*., 2022).

Gut microbiota research can be used as a basis for evaluating the quality of the captive animal environment (Mohd-Yusof *et al*., 2022; Khairulmunir *et al*., 2023). Gut microbiota status can reflect whether the animal has adapted to its diet or captive environment conditions (Amato *et al*., 2013; Campbell *et al*., 2020). Thus, identifying the factors influencing the gut microbiota of endangered species will aid in understanding their ecological adaptation and conservation management in captivity (Weng *et al*., 2016; Wu *et al*., 2017; Wiebler *et al*., 2018; Zhu *et al*., 2018). Recent advancements in next-generation sequencing (NGS) have enabled the analysis of gut microbiota using the 16S rRNA gene. This locus is widely used because it contains conservative and hypervariable regions that allow taxonomic identification and differentiation of organisms (Ames *et al*., 2017).

Several studies have been conducted to determine the association between gut microbiota in progenies and mothers. For example, Petrullo *et al*. (2022) investigated the influence of maternal parity on the gut microbiome of vervet monkeys (*Chlorocebus aethiops sabaues*). Their findings indicate that the gut microbiota of infant vervet monkeys is more similar to their mother's milk microbiota than to their mother's gut microbiota. Other studies on the rhesus macaque (*Macaca mulatta*) have found that breastfeeding is one of the most important factors influencing the development of the primate's gut microbiota during neonatal growth. Rhesus macaque progenies that receive breastmilk from their mothers have a higher abundance of beneficial commensal bacteria (*Bifidobacteria* and *Lactobacillus*) and acquire microbiota more quickly than their formula-fed counterparts (Rendina *et al*., 2019).

The white-handed gibbon locally known as ungka is a small ape that belongs to the family Hylobatidae. In Peninsular Malaysia, two subspecies can be found namely, *H. l. enteloides* (Northern Peninsular) and *H. l. lar* (from Sungai Perak to Southern Peninsular) (Md-Zain *et al*., 2022). Previous genetic research on Hylobatidae has primarily focused on genetic identification, taxonomy, and phylogenetic analysis (Md-Zain *et al*., 2010; Thinh *et al*., 2010a; b; Aifat & Md-Zain, 2021; Gani *et al*., 2021; Sariyati *et al*., 2024). However, research on the metabarcoding analysis in the gut microbiota of the Hylobatidae family is very limited, particularly in captive gibbons. The gut microbiota difference between adult and juvenile gibbons is significant given that age is known to influence gut microbiota diversity (Ying *et al*., 2022). Identifying gut microbiota composition will improve our understanding of nutritional adaptations and the health of white-handed gibbons in captivity (Jia *et al*., 2018). Gut microbiome studies have great potential in captive primate management and conservation efforts (Stumpf *et al*., 2016).

Previous primate metabarcoding studies have been conducted in Peninsular Malaysia; however, they are limited to the Cercopithecinae *Macaca arctoides* and *Macaca fascicularis* (Osman *et al*., 2020; 2022). Research on Bornean primate gut microbiota is restricted to Proboscis monkeys in Sabah, Malaysia (Hayakawa *et al*., 2018). The status of gut microbiota during the early growth period could determine progeny health. This process is strongly influenced by the maternal gut microbiota (Rendina *et al*., 2019). Thus, the main objective of this study was to determine the variations in gut microbiota in the mother and offspring of white-handed gibbons at Melaka Zoo using 16S rRNA NGS. Comparison between mother and offspring gut microbiota will improve our understanding of the health and adaptability of captive white-handed gibbons.

MATERIALS AND METHODS

Sample collection

This study focused on white-handed gibbons, a mother and its infant, at Zoo Melaka, Malaysia. Zoo Melaka made history as the first zoo in Asia to record the birth of twin white-handed gibbons (Figure 1) in August 2021 (Mulup, 2021; Yaakob, 2021). This study employed noninvasive sampling methods (Abdullah-Fauzi *et al*., 2022). Fecal samples were collected from the white-handed gibbon mother, named Sandy (sample code 917), and one of the twin infants, named Maru (sample code 918). During the time of sampling, Maru's twin had tragically passed away in an accident when Sandy was swinging from a tree. Therefore, only two fecal samples from Sandy (sample code 917) and Maru (sample code 918) were collected for this study. Maru was approximately one year old at the time the fecal sample was collected.

DNA extraction and amplicon preparation

Whole genomic DNA was extracted from the fecal samples of Sandy (sample code 917) and Maru (sample code 918) using the QIAamp® PowerFecal® Pro DNA Kit (QIAGEN, Germany), following the manufacturer's protocol with some modifications based on the laboratory equipment used (Aifat *et al*., 2016).

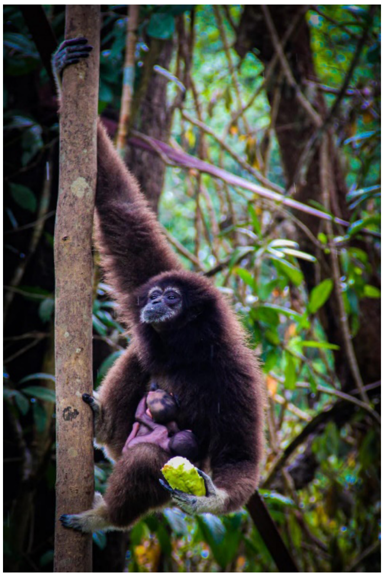

Fig. 1. White-handed gibbon mother with a twin baby at Zoo Melaka.

To assess the DNA concentration of the extracted DNA samples (Mohd-Radzi *et al*., 2022), a polymerase chain reaction (PCR) was performed using universal 16S rRNA gene bacterial primers. The amplification process was performed using a Thermocyler Prime machine (Techne) with PCR components and profiles based on the protocols by targeting the V3 and V4 regions as described by Mohd-Yusof *et al*. (2022) and Khairulmunir *et al*. (2023). Both samples were successfully amplified for the initial quality control test. Subsequently, the extracted DNA samples (sample code 917 for Sandy and sample code 918 for Maru) were sent to Apical Scientific Sdn. Bhd. to be prepared for amplicon sequencing. The library preparation process involved a two-stage PCR. For initial library construction, the extracted DNA samples were amplified using 16S rRNA V3–V4 primers with overhang adapters. In the second PCR stage, dual indices were attached to the amplicon PCR using the Nextera XT kit v2 according to the manufacturer's protocol. Subsequently, the libraries were normalized and proceeded to sequencing on the Illumina MiSeq platform with 300 paired-end sequencing.

Data analysis

The obtained sequences were filtered and processed using DADA2 V1.18 software to generate amplicon sequence variants (ASVs) (Callahan *et al*. 2016). The assessment of the quality of raw sequencing reads was conducted using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then, sequencing primers and adapters were removed using Cutadapt 3.5 (Martin, 2011). The paired-end reads were processed and merged using DADA2 V1.18 (Callahan *et al*., 2016). Subsequently, chimeric sequence assessment and taxonomic classification were performed using the SILVA nr V138 database. Statistical analysis of the ASV data was performed using RStudio. The relative abundance of gut microbiota in the gibbons was assessed using the ASV data and is illustrated in bar graphs and heat maps. Alpha diversity indices (Chao1, Shannon, and Simpson) (McMurdie & Holmes 2013) were used to determine the diversity and richness of gibbon gut microbiota. The differential abundance between Maru and Sandy was calculated using the Mann–Whitney *U* test in package stats of R (R Core Team, 2016). Venn diagrams were generated to identify the number of unique and shared ASVs between the two gibbon samples (Osman *et al*., 2022; Khairulmunir *et al*., 2023).

RESULTS

A total of 320,128 raw and 198,957 non-chimeric reads were successfully obtained (Table 1). There were 708 ASVs detected in both *H. lar* individuals, with 352 specific ASVs for Sandy and 415 for Maru. Table 1 summarizes the alpha diversity, which includes the Shannon (H′), Simpson (D), and Chao1 indices for both *H. lar* individuals. As shown in Table 1, Maru exhibited higher microbial diversity (H′ = 4.7591, D = 0.9814) than Sandy (H′ = 4.5437, D = 0.9776). In terms of the Chao1 index, Maru showed higher diversity (414.2) than Sandy (349.5). The differential abundance of the microbiome was supported by the Mann-Whitney test, indicating a significant *p*-value<0.05.

Table 1. Alpha diversity indices (Shannon (H′), Simpson (D), and Chao1) for both *H. lar* individuals

Sample	Non-chimeric Sequence	ASV	Shannon (H')	Simpson (D)	Richness (Chao1)
Sandy 917	93448	352	4.5437	0.9776	349.5
Maru 918	105509	415	4.7591	0.9814	414.2
Total	198957	708			
Mann-Whitney *U* test	*p*-value = 0.001973				

The ASVs were successfully assigned to 15 phyla, 49 orders, 69 families, and 134 genera of bacteria. This study found that Firmicutes has the highest relative abundance (44.98%), followed by Bactereroidota (43.49%). The other phylum bacteria recorded included Proteobacteria (4.27%), Spirochaetota (3.44%), Patescibacteria (1.22%), Elusimicrobiota (0.64%), and Desulfobacterota (0.57%). Cyanobacteria, Verrucomicrobiota, Actinobacteriota, WPS-2 (Eremiobacterota), Campylobacterota, Fibrobacterota, Thermoplasmatota, and Synergistota showed a relative abundance of less than 5%. Overall, the bacterial composition of Sandy and Maru was dominated by both Firmicutes and Bacteroidota, with an average abundance of 89.4%. At the genus level, both *Prevotella* (12.74%) and *Prevotella_9* (12.18%) were the most abundant bacteria in the two *H. lar* individuals (Table 2).

Table 2. Relative abundance of microbial communities at the phylum and genus levels in the two *H. lar* individuals at Zoo Melaka

Phylum	Relative abundance (%)	Genus	Relative abundance (%)
Firmicutes	44.98	*Prevotella*	12.74
Bacteroidota	43.49	NA	12.64
Proteobacteria	4.27	*Prevotella*_9	12.18
Spirochaetota	3.44	*Lachnospiraceae* AC2044	5.04
Patescibacteria	1.22	*Anaerovibrio*	4.56
Elusimicrobiota	0.64	*Oscillospiraceae* UCG-005	4.11
Desulfobacterota	0.57	*Lachnoclostridium*	3.51
Cyanobacteria	0.49	*Prevotellaceae* NK3B31	3.42
Verrucomicrobiota	0.31	*Treponema*	3.40
Actinobacteriota	0.24	*Phascolarctobacterium*	3.16
WPS-2	0.23	*Lachnospiraceae* NK4A136	2.69
Campylobacterota	0.05	*Eubacterium*	2.51
Fibrobacterota	0.04	*Subdoligranulum*	2.21
Thermoplasmatota	0.02	*Ruminococcus*	2.12
Synergistota	0.00	*Alloprevotella*	1.74

At the phylum level, Firmicutes had the highest relative abundance (47.56%) among the microbial communities in Sandy (mother *H. lar*), followed by Bacteroidota (38.60%) (Figure 2). In contrast, Bacteroidota was the highest (47.83%), followed by Firmicutes (42.71%), in Maru (progeny *H. lar*). From this study, it is concluded that the relative abundance of the microbial communities between the mother and the progeny is different. *Prevotella* from the phylum Bacteroidota had the highest relative abundance (13.24%), followed by Firmicutes with UCG-005 (6.89%) and Lachnoclostridium (6.96%) in Sandy (mother). The microbial communities of Maru (infant) showed that the genera *Prevotella_9* (18.47%) and *Prevotella* (12.29%) from Bacteroidota had the highest relative abundance.

The family *Prevotellaceae* from the phylum Bacteroidota showed the highest relative abundance, followed by *Lachnispiraceae* (Firmicutes) for both *H. lar* individuals (Table 3). However, the infant (Maru) exhibited a higher abundance of *Prevotellaceae*, whereas the mother (Sandy) exhibited a higher abundance of the family *Lachnispiraceae*.

Based on the Venn diagram, out of the 708 acquired ASVs, 59 shared ASVs were common in both individuals (Figure 3). Moreover, 356 ASVs were assigned uniquely to Maru, whereas only 293 ASVs were assigned uniquely to Sandy.

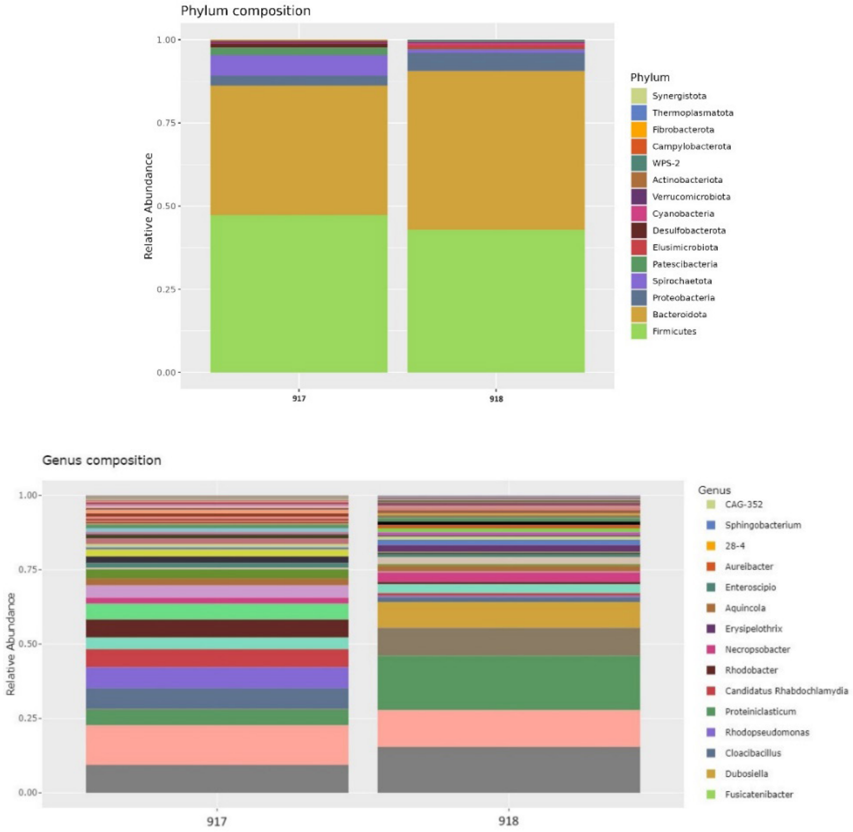

Fig. 2. Relative abundance of microbial composition at the phylum and genus levels in the two *H. lar* individuals

Table 3. The 15 most abundant family bacteria in the gut microbiome of the mother (Sandy) and infant (Maru) at Zoo Melaka

Family	Mother (%)	Family	Infant (%)
Prevotellaceae	28.96	Prevotellaceae	36.06
Lachnospiraceae	21.32	Lachnospiraceae	19.61
Oscillospiraceae	10.29	Selenomonadaceae	8.62
Ruminococcaceae	8.02	Muribaculaceae	7.48
Spirochaetaceae	6.19	Ruminococcaceae	4.28
Acidaminococcaceae	5.92	Oscillospiraceae	4.17
Muribaculaceae	4.01	NA	3.85
Saccharimonadaceae	2.34	Sutterellaceae	3.58
Moraxellaceae	1.76	Bacteroidales RF16	1.90
Bacteroidaceae	1.63	Rikenellaceae	1.19
Rikenellaceae	1.59	Lactobacillaceae	1.17
Bacteroidales RF16	1.30	Elusimicrobiaceae	1.14
Desulfovibrionaceae	1.16	Bacteroidaceae	1.05
Eubacterium coprostanoligenes	0.79	Spirochaetaceae	1.00
Tannerellaceae	0.64	Acidaminococcaceae	0.72

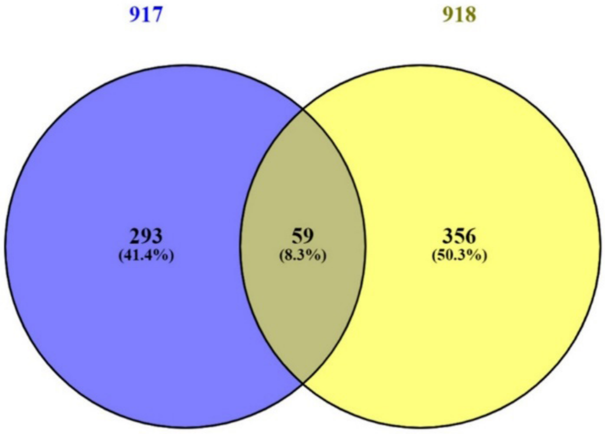

Fig. 3. Venn diagram of unique and shared ASVs detected between Maru (sample code 918) and Sandy (sample code 917)

DISCUSSION

In total, 15 phyla of bacteria were successfully assigned to 68 families and 133 genera from the ASVs of the two *H. lar* individuals. The research approach through the NGS technique has provided more detailed microbial composition than conventional techniques (Wang *et al*., 2015). The analyses of relative abundance show that the gut microbiome of *H. lar* in enclosures is dominated by the phyla Firmicutes and Bacteroidota. This finding is congruent with a previous study on the gut microbiome of captive *H. lar* (Ying *et al*., 2022). Furthermore, both Firmicutes and Bacteroidota are prevalent in other captive gibbon species such as the Hainan gibbon (*Nomascus hainanus*) (Li *et al*., 2022) and Northern white-cheeked gibbon (*N. leucogenys*) (Jia *et al*., 2018). A recent study on the gut microbiome conducted on genera *Nomascus*, *Hylobates*, *Hoolock*, and *Symphalangus* also revealed that both Firmicutes and Bacteroidota dominated the microbial composition (Lan *et al*., 2022). Both phyla play important roles in fermentation and nutrient assimilation from plant-based food sources (Ley *et al*., 2008; Zhang *et al*., 2016; Borbón-García *et al*., 2017).

Both *Prevotella* and *Prevotella_9* from the phylum Bacteroidota showed the highest relative abundance at the genus level among the study individuals. Each microbial genus within the gut encompasses several strains or subtypes, which exhibit significant variations in their genetic makeup and impart different functional capabilities (Greenblum *et al*., 2015; De Filippis *et al*., 2019). The genus *Prevotella* is commonly detected in other primates such as Cercopithecinae, as this bacterium is essential in the fermentation of simple sugars and carbohydrates in fruits (Amato *et al*., 2015). *Prevotella* is capable of fermenting polysaccharides from a high intake of fibers and carbohydrates in plants (Clayton *et al*., 2016; Gomez *et al*., 2016; Precup & Vodnar, 2019). Gibbons are frugivorous, and 50% of their daily diet includes young leaves, insects, and flowers (Stevenson *et al*., 2000; Simmen *et al*., 2003).

A genus from the family *Lachnospiraceae* (e.g., *Lachnospiraceae* AC2044) is known to be dominant in the gut microbiome and is important in maintaining the gut's health (Vojinovic *et al*., 2019). The bacterial families *Lachnospiraceae* and *Ruminococcaceae* are essential in producing short fatty acid chains (Ma *et al*., 2020) to regulate the immune function of body cells (Corrêa-Oliveira *et al*., 2016; Laforest-Lapointe & Arrieta, 2017). Other animals such as sheep rely on these bacteria during the early development of their immunity (Yin *et al*., 2023).

Genus *Treponema* is one of the 15 most abundant bacteria in this study. Generally, *Treponema* consists of both pathogenic and nonpathogenic bacteria. There are two species of nonpallidum *Treponema* (nonpathogenic) were successfully identified, namely, *T. succinifaciens* and *T. berlinense*, whereas other *Treponema* spp. were not able to be identified at the species level. *T. succinifaciens* is commonly found in humans (Angelakis *et al*., 2019), whereas *T. berlinense* can be found in the gut microbiome of a pig (Nordhoff *et al*., 2005). Although there are still limitations in identifying bacteria at the species level through DNA metabarcoding of the gut microbiome, this analysis can be used as a guide for the implementation of culturing more specific pathogenic bacteria species for diagnostic purposes.

The gut microbiota composition of mother and infant white-handed gibbons differs at the phylum, family, and genus levels. Sandy (mother) had the greatest abundance of bacteria from the Firmicutes phylum, whereas Maru (infant) had the greatest abundance of bacteria from the Bacteroidota phylum. These findings are consistent with those of Li *et al*. (2022), who discovered that the Firmicutes bacterial composition increases with gibbon age, whereas the Bacteroidota bacterial composition declines. According to Li *et al*. (2022), this could be explained by the immature immune system of juvenile gibbons, which is still developing, as well as the unstable gut microbiota. Bacteria such as *Bacteroides* can boost the immune system, hence raising immunity levels (Hooper, 2004).

Bacteroides play an important role in the gastrointestinal tract of young gibbons. At Zoo Melaka, there was no substantial change in the relative abundance of the *Bacteroides* between adult and juvenile gibbons. Although the value of the relative abundance of Bacteroides in this study is found to be lower than in the *Nomascus hainanus* gibbons' study by Li *et al*. (2022), no significant comparisons were made in this matter due to differences in sample size. However, Lan *et al*. (2022) discovered that *Bacteroides* was found to be more abundant in *Nomascus* primates than in *Hylobates* primates. Furthermore, *Bacteroides* are reported to be lower in captive primates; as observed in captive *H. lar* rather than in wild *H. lar* gibbons (Ying *et al*., 2022).

The relationships between the phyla Bacteroidota and Firmicutes and gibbon age are linked to changes in the physiological processes of digestion in adult and young gibbons. This is because the diet of young gibbons switches toward fruits, vegetables, and grains as their age increases. Adult gibbons have a larger relative abundance of the phylum Firmicutes than young gibbons because of the increasing ingestion of high-fiber diets (Jia *et al*., 2018). Maru has a larger abundance of bacteria from the *Prevotella* group than her mother. This finding agrees with that of Li *et al*. (2022), who discovered that young *N. hainanus* gibbons have a larger abundance of *Prevotella* than adult gibbons. As their daily intake includes of mix of leafy vegetables, starchy vegetables, fruits, and boiled eggs or boiled chicken, this would explain the presence of a high abundance of *Prevotella* in their gut microbiome. *Prevotella* is essential for the digestion and utilization of hemicellulose, pectin, starch, and mucopolysaccharides (Flint, 2004) whereby all of these nutrient elements can be found in their diet. This is linked to the ingestion of high-pectin fruits in their diet. This shows that juvenile gibbons are better equipped to digest unstructured carbohydrates such as fruits, whereas adult gibbons are better adapted to digest structured carbohydrates found in leaves (Li *et al*., 2022).

According to analyses of diversity indices, Maru has a greater diversity and abundance of gut microbiota than her mother Sandy. These findings contradict those of Jia *et al*. (2018), who reported that the richness and diversity of bacterial communities were greater in adult *N. leucogenys* than in juveniles living in the same habitat. The diversity of gut microbiota in the gastrointestinal tract is linked to better digestive performance. However, Reese *et al*. (2021) discovered that the gut microbiome diversity in young chimpanzees is greater than that in adult chimpanzees. Their findings support Maru having higher microbial diversity and abundance than Sandy. Reese *et al*. (2021) proposed that the great diversity of gut microbiota in young chimpanzees is related to their immune system which has yet to reach maturity stage. The classification of young primate here refers to the age < 2 years old (Maru: 1 year old). Juvenile primates have less mature immune systems than adults, allowing simpler bacterial colonization in the gut microbiota (Reese *et al*. 2021). However, in young primates that continue to consume milk, oligosaccharides from maternal milk can limit the colonization of pathogenic bacteria in the gut microbiota (Reese *et al*. 2021).

Low bacterial diversity has been linked to host illness, whereas higher diversity often implies better health (Scott *et al*. 2015). Several studies have shown that physiological and psychological stress can affect the gut microbiota of mammals, including rodents (Sun *et al*. 2019) and primates (Bailey & Coe, 1999; Bailey *et al*., 2004; Zheng *et al*., 2021). Bailey and Coe (1999) discovered that after being separated from their mothers, young rhesus monkeys (*M. mulatta*) had a significant decrease in gut microbial diversity.

Zheng *et al*. (2021) discovered that long-tailed macaques (*M. fascicularis*) exhibiting depressive behavior exhibited lower alpha diversity indices (Chao1 and ACE) than the control group, with no significant difference in Shannon index values between the two groups. Ghosh *et al*. (2021) found that increasing stress in captive *Macaca munzala* resulted in reduced alpha diversity indices in their gut microbiota. These two studies discovered that the number of particular bacterial taxa is highly associated with stress observed in primates. The lower level of gut microbiota diversity observed in Sandy at Zoo Melaka is may probably due to the stress she experiences after losing her second twin offspring. Nevertheless, the correlation between low gut microbial diversity and distress level in Sandy due loss of one of its offspring remains postulated. This study has limitations as no hormonal study was conducted such as cortisol test levels in blood to support or refute the hypothesis.

CONCLUSION

This study demonstrates that NGS with DNA metabarcoding allows for a more in-depth analysis of the gut microbiota communities of primates in captivity. Firmicutes and Bacteroidetes dominate the gut microbiome community of captive white-handed gibbons. The gastrointestinal system of gibbons is dominated by *Prevotella* bacteria at the genus level, and *Prevotellaceae* and *Lachnospiraceae* are prominent at the family level. Despite being raised in the same enclosure, the mother and infant had different abundances of gut microbiota communities at the phylum, family, and genus levels. However, we acknowledged due to the limitation of this study i.e. small sample size and lack of alternative tests; the correlation between Sandy's postcondition on her stress level after the loss of its infant and low microbial diversity compared to Maru remains postulated. Indeed, the findings of this study can be used as an indicator to address the health and nutritional needs of the captive white-handed gibbons. Understanding the composition of the gut microbiota and how it alters between adults and offspring will help researchers better understand their digestive health and overall well-being. The zoo can make informed judgments about the gibbons' meals and implement actions to improve their health and ensure they receive the necessary nutrition for their particular life phases by analyzing their microbiome profiles. This can help improve the overall well-being and conservation efforts of small apes in captivity.

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ETHICAL STATEMENT

Research methods reported in this manuscript adhered to the legal requirements of Malaysia and were approved by the Department of Wildlife and National Parks (PERHILITAN), Peninsular Malaysia, Malaysia under research permit JPHL&TN(IP):100-34/1.24 Jld 19 (14.4).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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