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Gut microbial assessment among Hylobatidae at the National Wildlife Rescue Centre, Peninsular Malaysia

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ABSTRACT

Importance: Recent developments in genetic analytical techniques have enabled the comprehensive analysis of gastrointestinal symbiotic bacteria as a screening tool for animal health conditions, especially the endangered gibbons at the National Wildlife Rescue Centre (NWRC).
Objective: High-throughput sequencing based on 16S ribosomal RNA genes was used to determine the baseline gut bacterial composition and identify potential pathogenic bacteria among three endangered gibbons housed in the NWRC.
Methods: Feces were collected from 14 individuals (*Hylobates lar*, n = 9; *Hylobates agilis*, n = 4; and *Symphalangus syndactylus*, n = 1) from March to November 2022. Amplicon sequencing were conducted by targeting V3–V4 region.
Results: The fecal microbial community of the study gibbons was dominated by Bacteroidetes and Firmicutes (phylum level), Prevotellaceae and Lachnospiraceae/Muribaculaceae (family level), and *Prevotella* (and its subgroups) (genera level). This trend suggests that the microbial community composition of the study gibbons differed insignificantly from previously reported conspecific or closely related gibbon species.
Conclusions and Relevance: This study showed no serious health problems that require immediate attention. However, relatively low alpha diversity and few potential bacteria related to gastrointestinal diseases and streptococcal infections were detected. Information on microbial composition is essential as a guideline to sustain a healthy gut condition of captive gibbons in NWRC, especially before releasing this primate back into the wild or semi-wild environment. Further enhanced husbandry environments in the NWRC are expected through continuous health monitoring and increase diversity of the gut microbiota through diet diversification.

Keywords: Gibbons; high-throughput nucleotide sequencing; 16s ribosomal RNA; microbial community

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Conflict of Interest

The authors declare no conflicts of interest.

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INTRODUCTION

Many primate species are threatened with extinction due to human activities [1]. Persistent *ex situ* conservation efforts in zoo animal husbandry have improved the reproductive success and well-being of zoo animals [2]. Different approaches have been developed, including examining diet regimes [3,4], analyzing the shape of feces and their nutrients [5], and measuring hormone levels in feces [6]. Recent developments in genetic analytical techniques with the relatively facilitated and lower cost of a comprehensive analysis of gastrointestinal symbiotic bacteria, including the determination of their composition and the specific bacterial community involved in diseases, are now being used to screen for animal health conditions [7].

Gibbons (Hylobatidae), also known as lesser apes, are hominoids [8] that generally live in forests in certain parts of South and Southeast Asia [9]. Hylobatidae can be categorized into four genera: *Nomascus*, *Hoolock*, *Hylobates*, and *Symphalangus* [9]. In Malaysia, there are five species from two genera, i.e., *Hylobates* and *Symphalangus*, all of which are endangered species [10]. In the Malaysian peninsula, National Wildlife Rescue Centre (NWRC), located in Sungkai, has been set up and designed as a sanctuary for rescued wildlife species due to injury or confiscation/illegal trading [11] including these endangered gibbons. The center is involved in breeding programs with other wildlife zoos in Malaysia, with the aim of conserving wildlife populations. Dietary, health, behavioral, and social rehabilitation is a standard practice for all captive animals [12], including gibbons, at the NWRC to assess their condition. It is a framework for retaining and restoring the natural behavior of these animals in captivity before relocating or translocating them into the wild.

Every individual has a distinct microbial community that influences their nutrition, metabolism, and immunity [13]; even within conspecifics, depending on its environment and diets [14,15]. As the gut microbiome diversity is associated with the overall better health and immune function [16], maintaining gut microbiome diversity similar to the wild is especially important in captive primate management. This prior strategy is significantly important before releasing these captive primates back into their wild environment [17]. Primates with varied gut microbiome has less risk towards diseases and are better to adapt to their surroundings. Therefore, this study aimed to comprehensively analyze the gut microbiota of *Hylobates lar*, *Hylobates agilis,* and *Symphalangus syndactylus*, through high-throughput sequencing based on 16S ribosomal RNA genes, and further identify potentially pathogenic bacteria, at the NWRC to have better understanding its role in affecting their health in enclosures.

METHODS

Study site and fecal sampling

Research methods applied in this study adhered to the legal requirements of Malaysia and was approved by Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN), Peninsular Malaysia, Malaysia under research permit JPHL&TN(IP):100-34/1.24 Jld 19 (14.4). Fecal samples were collected from 14 individuals representing all three species inhabiting Peninsular Malaysia (*H. lar*, n = 9; *H. agilis*, n = 4; *S. syndactylus*, n = 1) at the National Wildlife Rescue Centre (NWRC) located in Sungkai, Perak, Malaysia. The collection of fecal samples was non-invasively conducted in NWRC enclosures from March 2022 to November 2022 (**Table 1**). All collected fecal samples were labeled and preserved in absolute ethanol following the protocol described by Aifat and Md-Zain [18] and then stored in a freezer at −20°C.

Table 1. Individuals of Hylobatidae and the type of enclosure in the NWRC

#	Species	Accession number	Individual	Sex	Age	Type of enclosure	Fecal collection date
1	*H. lar*	SRR26378142	Intan	F	Adult	Exercise Yard	28-Mar-22
2		SRR26378196	Luka	M	Adult	Exercise Yard	28-Mar-22
3		SRR26378447	Bone	F	Adult	Nightstall	28-Mar-22
4		SRR26378615	Revi	M	Adult	Exercise Yard	29-Mar-22
5		S704RR26378	Manja	M	Adult	Pulau Ungka	1-Nov-22
6		SRR26378738	Chantiq	F	Adult	Pulau Ungka	1-Nov-22
7		SRR26378848	Kakak	F	Adult	Pulau Ungka	1-Nov-22
8		SRR26378851	Adik	M	Juvenile	Pulau Ungka	1-Nov-22
9		SRR26378866	Langat	F	Adult	Nightstall	1-Nov-22
10	*H. agilis*	SRR26379079	Awe Krai	M	Juvenile	Cage	1-Nov-22
11		SRR26379126	UTH 1	-	Adult	Exercise Yard	1-Nov-22
12		SRR26379316	UTH 2	-	Adult	Exercise Yard	1-Nov-22
13		SRR26379362	Burn	M	Adult	Nightstall	28-Mar-22
14	*S. syndactylus*	SRR26379370	Bilut	F	Subadult	Nightstall	1-Nov-22

NWRC, National Wildlife Rescue Centre.

DNA extraction and sequencing

DNA from all samples was extracted using the QIAampPowerFecal Pro DNA Kit following the manufacturer's standard protocol. Genomic DNA concentration was measured for quality and quantity using an Implen Nano Photometer [19]. Qualified products of Genomic DNA were sent to Apical Scientific Sdn Bhd for further sequencing. Amplicon sequencing of the gut microbiome was performed by amplifying the 16S rRNA gene (V3–V4 region) [20] using the following primers: forward primer, F515 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCG CGGTAA-3′) and reverse primer, R805 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACHVGGGTWTCTAAT-3′).

Analyses

Sample visualization and data analyses were conducted using R studio with DADA2 packages [21]. FASTQ files were used to assess the quality scores of the Illumina data. Amplicon sequence variants (ASVs) were clustered with 97% similarity. ASVs were aligned using the MUSCLE tool in GENEIOUS. Compared with ASV, phylogenetic classification was performed using the GenBank database for 16S microbiome studies. Besides the overall trends in the gut microbiota, the following bacteria associated with gastrointestinal disorders (i.e., *Acidaminococcus*, *Bulleidia*, *Campylobacter*, *Escherichia*, *Eubacterium*, *Megasphaera*, *Pastuerella*, *Phascolarctobacterium*, *Selenomonas*, *Shigella*, *Streptococcus*, *Succiniclasticum*, *Succinovibrio*, *Vibrio*, and *Yersinia*), which have been documented in previous primate gut microbiome studies [7,22], were also examined for their abundance in this study. Alpha (Observed richness, Shannon, and Chao 1) and beta diversity were calculated using the R package statistical functions and *phyloseq*. Principal coordinate analysis (PCoA) based on weighted unifrac and the Bray-Curtis distance method [23] was used to compare the bacterial composition of the samples. To compare the alpha diversity between *H. lar* and *H. agilis*, the Wilcoxon rank-sum test was used; note that the *S. syndactylus* data was excluded because of the limited number of samples. PERMANOVA analysis was conducted to measure the effect size and significance of beta diversity among *H. agilis*, *H. lar,* and *S. syndactylus*. A Venn diagram was generated to identify the number of shared and unique ASVs among the samples. Heat maps were constructed based on the Bray-Curtis distances to assess the relationship between bacterial communities and primate samples. All analyses were conducted using R ver. 4.3.0 (R Core Development Team, USA; 2023), and a significant difference was set at $p < 0.05$.

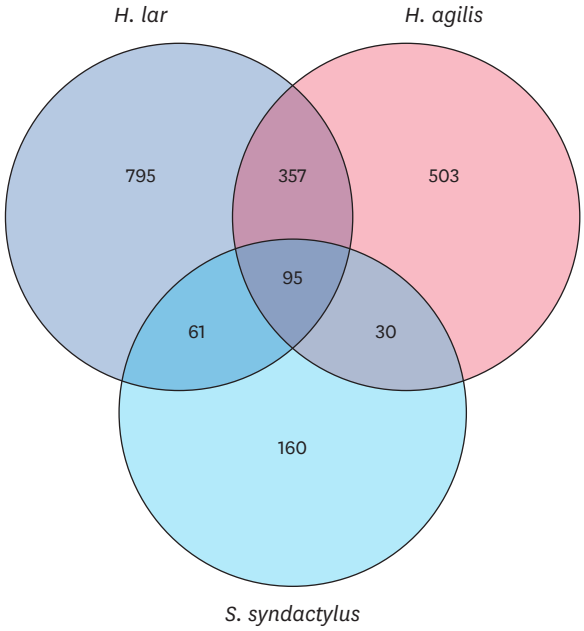

Fig. 1. Venn diagram of unique and shared ASVs among the three species of Hylobatidae.
ASV, amplicon sequence variant.

RESULTS

Quality assessment of sequencing data

In 14 fecal samples, 1,946,592 raw and 1,238,212 non-chimeric reads were successfully obtained. Of the 1,238,212 reads, 64,870 rarefied reads were obtained to avoid bias in the analyses, and 2,001 ASVs were detected in all fecal samples, which were classified into 20 phyla, 34 classes, 101 families, 230 genera, and 52 species. There were 795 ASVs specific for *H. lar*, 503 for *H. agilis*, and 160 for *S. syndactylus*, with 95 ASVs shared by the three species (**Fig. 1**).

Bacterial composition

Overall

At the phylum level, the top five taxa, i.e., Bacteroidota (50.4%–67.5%), Firmicutes (19.5%–38.4%), Spirochaetota (5.6%–6.9%), Proteobacteria (4.0%–5.2%), and Cyanobacteria (0.9%–1.7%), were consistent across the three species, except for *S. syndactylus*, in which the fifth was not a Cyanobacteria but a Verrucomicrobiota (**Table 2**). At the family and genus levels, the top five patterns of the gut microbial community across the species were also relatively consistent, albeit with some differences. Prevotellaceae and Lachnospiraceae were prevalent in *H. lar* and *H. agilis*. Meanwhile, Muribaculaceae and Prevotellaceae were predominant in *S. syndactylus* (**Table 2**). At the genus level, the gut microbial community was dominated by *Prevotella_9* (12.95%), followed by *Treponema* (7.08%), *Prevotellaceae UCG-001* (5.87%), *Prevotella* (5.08%), *Rikenellaceae RC9* gut group (4.17%), *Alloprevotella* (3.61%), *Faecalibacterium* (2.52%), *Ruminococcus* (2.51%), *Prevotellaceae NK3B31* group (2.11%), *Lachnospiraceae NK3A20* group (1.56%), and unknown genera (18.75%) (**Table 2**).

Bacteria potentially associated with gastrointestinal disorders

Of the 15 targeted bacteria potentially associated with gastrointestinal disorders, nine were

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Table 2. Top 10 most abundant bacterial compositions in three different Hylobatidae species at the phylum, family, and genus levels

Bacterial compositions	*H. lar* (%)	*H. agilis* (%)	*S. syndactylus* (%)
Phylum			
Bacteroidota	50.42	50.78	67.50
Firmicutes	32.58	38.40	19.48
Spirochaetota	6.68	6.93	5.63
Proteobacteria	5.21	4.79	4.00
Cyanobacteria	1.74	1.47	0.92
Desulfobacterota	1.11	0.91	0.01
Verrucomicrobiota	0.51	0.99	1.72
Actinobacteriota	0.72	0.70	0.06
Fibrobacterota	0.38	0.35	0.28
Elusimicrobiota	0.19	0.19	0.10
Family			
Prevotellaceae	34.87	25.85	26.31
Lachnospiraceae	12.98	14.50	7.69
Muribaculaceae	8.34	7.86	34.18
Ruminococcaceae	8.09	9.41	2.53
Spirochaetaceae	6.68	8.77	5.63
Rikenellaceae	3.70	5.92	3.90
Oscillospiraceae	2.57	6.47	3.85
Succinivibrionaceae	3.15	0.18	0.09
Sutterellaceae	1.06	2.66	3.25
Bacteroidales RF16 group	1.79	0.47	1.59
Unknown	4.70	2.56	3.62
Genus			
Prevotella_9	12.92	14.20	8.33
Treponema	6.60	8.56	5.57
Prevotellaceae UCG-001	8.16	1.02	4.63
Prevotella	4.98	4.33	8.90
Rikenellaceae RC9 gut group	3.63	5.56	3.52
Alloprevotella	4.30	2.35	2.43
Faecalibacterium	1.81	4.50	1.10
Ruminococcus	2.37	3.34	0.54
Prevotellaceae NK3B31 group	2.26	1.80	1.99
Lachnospiraceae NK3A20 group	1.92	1.16	0.00
Unknown	18.32	13.80	41.58

Table 3. Detected bacteria potentially associated with gastrointestinal disorders in the study species

Detected bacteria	*H. lar*	*H. agilis*	*S. syndactylus*
Acidaminococcus	27	0	0
Campylobacter	466	180	33
Eubacterium	5,965	2,088	159
Escherichia-Shigella	182	26	0
Megasphaera	366	0	0
Phascolarctobacterium	1,179	1,462	76
Selenomonas	4,547	287	0
Streptococcus	311	34	0
Succinivibrio	18,406	620	84
Total	31,449	4,697	352

Values are presented as number of bacteria detected.

found in the study animals: *Acidaminococcus*, *Campylobacter*, *Escherichia-Shigella*, *Eubacterium*, *Megasphaera*, *Phascolarctobacterium*, *Selenomonas*, *Streptococcus*, and *Succinovibrio* (**Table 3**). These bacteria were found more frequently in *H. lar* than in *H. agilis* and extremely less frequently in *S. syndactylus* than in *H. lar* and *H. agilis*. Among these detected bacteria, *Campylobacter*, *Eubacterium*, *Phascolarctobacterium*, and *Succinivibrio* were found across the three species, although the frequency of the bacteria detected in each species varied.

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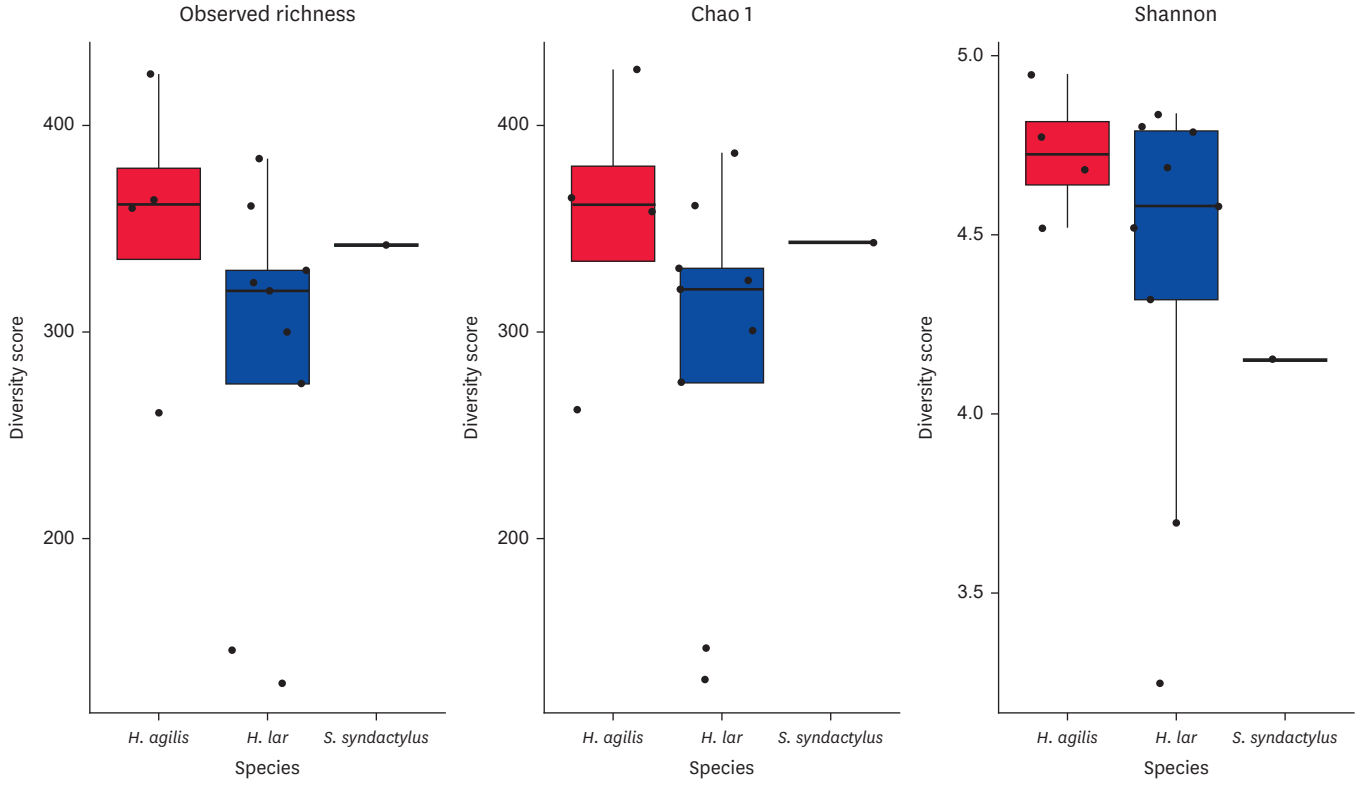

Fig. 2. Alpha diversity of the gut microbiome in *H. lar*, *H. agilis*, and *S. syndactylus*.

Bacterial diversity

Alpha diversity

Overall, the alpha diversity of the microbial community was higher in *H. agilis* (**Fig. 2**). However, the metrics between *H. agilis* and *H. lar* differed insignificantly, i.e., observed richness (U = 26; $p = 0.2472$), Chao 1 (U = 26; $p = 0.2472$), and Shannon (U = 23.5; $p = 0.4398$); the differences between *S. syndactylus* and other species could not be fully evaluated due to the limited sample size (n = 1). Relationship among sex, age and gut microbial community are observed to be insignificant ($p > 0.05$).

Beta diversity

The composition of the gut microbiome in Hylobatidae overlapped, as shown in the PCoA plot based on weighted UniFrac with Bray-Curtis (**Fig. 3A**); the first and second axes explained 20.3% and 12.7% of the total variation, respectively. There was no significant difference among the Hylobatidae, supported by Bray-Cutis PERMANOVA, F = 1.17; $p > 0.05$). The constructed heat map showed all genera that were used to determine the relationship of Hylobatidae (*H. lar*, n = 9; *H. agilis*, n = 4; *S. syndactylus*, n = 1) using weighted pair clustering based on Bray-Curtis (**Fig. 3B**). In the heat map, as the red color gets darker, it indicates the increasing abundance of the gut microbiome in Hylobatidae, whereas the blue color indicates a lower abundance of the gut microbiome.

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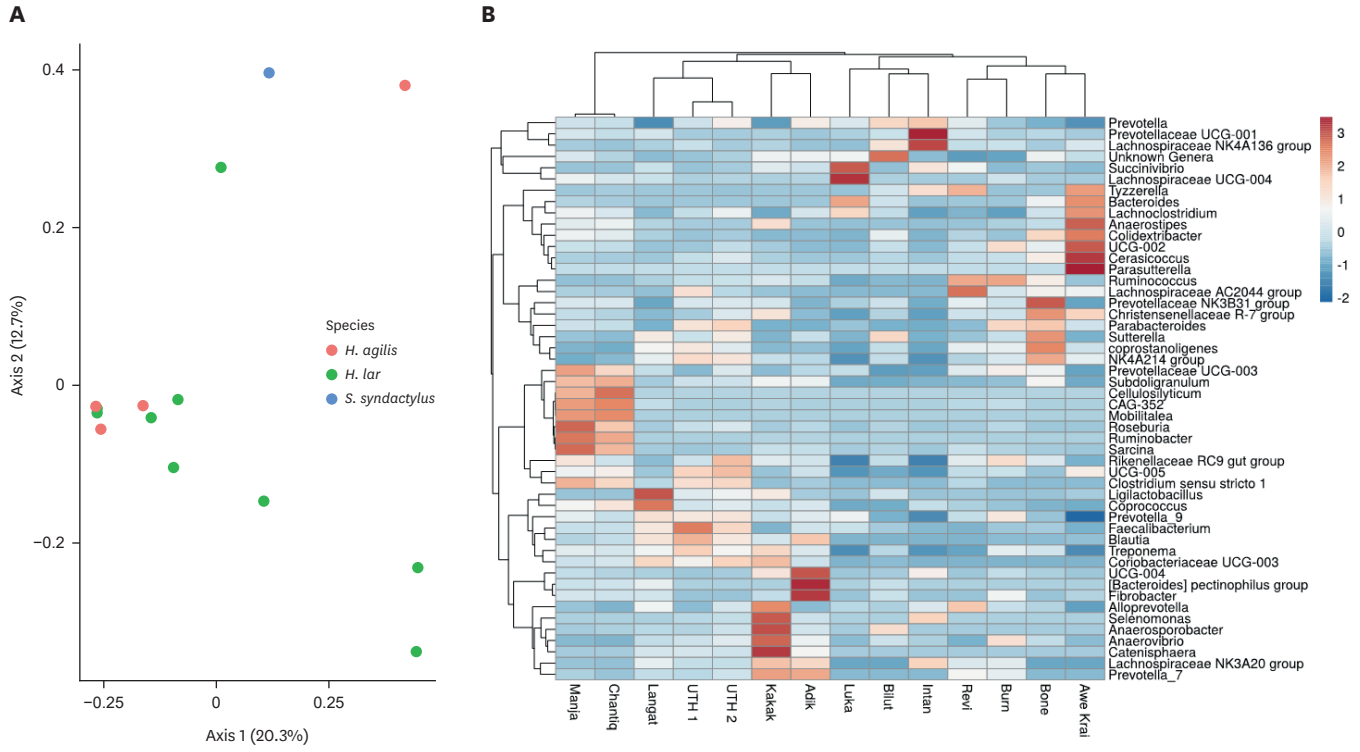

Fig. 3. Beta diversity of the gut microbiome of *H. lar* (n = 9, green), *H. agilis* (n = 4, red), and *S. syndactylus* (n = 1, blue). Diversities were analyzed based on (A) a principal coordinate analysis plot with weighted UniFrac distance and (B) a clustering of heat map for species richness at the genera level.

DISCUSSION

A comprehensive analysis of the gut microbiota of three species of gibbon, i.e., *H. lar*, *H. agilis,* and *S. syndactylus*, housed at the NWRC, was conducted to determine their composition and diversity. However, in this study, no significant differences were found in the overall gut microbial community composition among the three gibbon species. Furthermore, both sex and age does not influence the microbial community of gibbons in this study. Gut microbial composition typically changes throughout their lifetime, particularly during infancy and at later age again [24]. As solid foods are then introduced into the young captive gibbons, with the same diet as the adults; thus, this explains why the young gibbons of this study naturally will have a similar gut microbial composition with the adults in captivity.

The gut microbial composition also showed trends similar to those reported in previous studies of gibbon species (*H. lar* and *S. syndactylus*) [25,26] and other closely related gibbon species such as *Hoolock* and *Nomascus* [27-30]. The trends were especially similar at the phylum level, i.e., Firmicutes, Bacteroidetes, Spirochaetota, and/or Proteobacteria are generally included in the top five abundant phyla, and at the family level, i.e., Prevotellaceae, Lachnospiraceae, and Ruminococcaceae are in the top five abundant families, irrespective of the living environment either in captivity or the wild [25-30]. Conversely, at the genus level, the *Prevotella_9* and *Rikenellaceae RC9* gut groups, which were among the top five genera in this study, are only reported as top genera in *H. lar* and *S. syndactylus* kept at the Taiwan zoo [26]. Likewise, *Prevotella*, although a different gibbon species, has been reported as the dominant genus from *Nomascus leucogenys* in captivity at the Beijing Zoo [27] and *Nomascus hainanus* in the wild [28], respectively. Thus, the genus *Prevotella* (and its subgroups) is generally relatively

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predominant in captive gibbons, which also agrees with the general trend that *Prevotella* is commonly found in captives rather than in wild non-human primates [22,31]. *Prevotella* facilitates the fermentation of simple sugars and carbohydrates commonly found in the diet of captive animals, such as fruits, cereals, and pellets [31]. Indeed, at the NWRC, the study animals were typically fed winter melon, banana, apple, mango, longan, long bean, and maize as part of their daily diet, consistent with a *Prevotella*-dominated dietary environment.

No significant differences were found in the alpha diversity of the gut microbial community among the three study species; the statistical differences could not be examined for *S. syndactylus* because of the number of samples. Additionally, significant differences in beta diversity were not observed among the study species, suggesting that different gibbon species possess no distinctive microbiota signatures. These findings support the earlier study indicating that the gut microbiota among captive primates tends to be more similar to that of their natural habitat because of the uniformity of diets in captivity [32]. Conversely, as for the alpha diversity, Shannon's diversity index values for *H. lar* and *S. syndactylus* in this study were 3–5 and < 4.5, respectively, and considering that values of 5–6 have been reported for both species in other captive populations [26,28], the alpha diversity of the gibbons in this study would be rather lower. However, another captive species (*Nomascus* spp.) exhibited a wide range of Shannon's diversity index values (4–6.5) [27,28,30], suggesting that the alpha diversity of our study species might not be particularly lower than those of other gibbon species. Alpha diversity of gut-intestinal microbiota is often correlated with dietary diversity in primates [14,15]. Thus, the alpha diversity in the study animals could be increased by feeding a more diverse range of diets. Given that decreased gut bacterial diversity in primates may be linked to reduced functional redundancy and protection from potential gut pathogens [33], increasing alpha diversity through more diverse diets may enhance better captive environments for gibbons in the NWRC.

Our study revealed that the general trends in the gut microbiota of the study gibbons differed insignificantly compared with previously reported conspecific or closely related species, whereas many bacterial genera that have been suggested to be associated with gastrointestinal disorders were also detected. In Amato et al. [7], individuals with gastrointestinal disorders in captive foregut-fermenting douc langurs (*Pygathrix nemaeus*) had an excessive abundance of 11 bacterial genera, suggesting that they are associated with such disorders. A comprehensive review of the gut microbiota of non-human primates by Clayton et al. [22] also suggested that in addition to these 11 genera, *Escherichia*, *Shigella*, *Vibrio,* and *Yersinia* are also bacterial genera associated with gastrointestinal disorders. Of those 15 genera, nine were also detected in this study (**Table 3**); particularly, the four genera *Campylobacter*, *Eubacterium*, *Phascolarctobacterium,* and *Succinivibrio* were detected in all three studied species. These bacteria are likely to negatively affect the health status of captive animals, including those suggested to be associated with diarrhoeal illness in non-human primates, such as *Campylobacter*, *Escherichia*, and *Shigella* [22,34], as well as those associated with streptococcal infections, such as *Streptococcus* [35]. In contrast, some bacteria have no such clear negative impact. Some bacteria are even useful in maintaining healthy conditions, for example, *Eubacterium*, which has been suggested to play essential roles in modulating inflammation regulation of immune responses, maintaining barrier integrity in the gut, moderating glycemic response, and cholesterol homeostasis [36]. Rather than simply annotating the abundance of these bacteria, their overall balance may be essential in assessing the health status of captive individuals.

In addition to the bacterial genera that cause the potentially unhealthy gut microbial community described above, a recent study on captive gibbons in China supported our findings with *Treponema* as a very abundant bacteria compared with wild gibbons [37]. High levels of *Treponema* found in the gut across the three study species may indicate a dormant or underlying health problem [37] among the captive gibbons at NWRC. As an opportunistic pathogen, *Treponema* may contribute to dysbiosis of the gut microbiome, leading to the development of liver disorders. *Prevotellaceae-UCG-001,* which is abundant in all of the studied species, was previously discovered to be related to adenovirus (AdV) and helminth infection status, providing correlative proof of the major histocompatibility complex (MHC)'s indirect influence on the microbiome [38].

Gut microbiome assessment can be used as a standard pre-diagnostic health test for the host [17], particularly, for primates that live in captivity or having close contact with humans. Gaining more insight into the relationship between diet and health in primates and their microbial communities is crucial for understanding primate ecology, but it may also have significant effects on using non-human primates as model systems for altered lifestyles and related microbial changes [14]. Captive primates basically undergo dietary modifications or restrictions, antibiotic use or other veterinary medical interventions, drastically reduced ranges, decreased interactions with different habitat types, decreased interactions with other species, and increased exposure to microbes linked to humans [39]. All of the stated may affects the gut microbiome composition. Hence, as their gut microbiome composition is less diverse than those in the wild, it may lead these captive primates to be more vulnerable towards any pathogens of gastrointestinal disorders [7]. Significant differences in the gut microbiome between the captive and the wild individual may also introduces novel pathogens into the already susceptible wild populations [17]; if the assessment is not monitor prior relocation or translocation process either to the wild or semi-wild habitat.

Overall, this comprehensive screening of the gut microbiota study presented congruent findings with the previous microbial composition community of captive gibbons in other regions. It can be concluded that no health issue was significant to require immediate attention among the study gibbons in NWRC. However, relatively low alpha diversity and few bacteria that can cause gastrointestinal disorders were detected in this study. Previous information from this study can be used as a guideline to maintain a healthy gut condition of captive gibbons in NWRC, especially before releasing this primate back into the wild or semi-wild environment. Efforts to maintain activities, including various rescue operations, primate group rehabilitation, and recovery at the NWRC, and an increase in dietary diversity and health check-ups of captive individuals through regular gut microbiota screening, will lead to better captive environment improvement in the future.

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