ISSN 0026-2617, Microbiology, 2024, Vol. 93, No. 6, pp. 904–915. © Pleiades Publishing, Ltd., 2024.

EXPERIMENTAL ARTICLES

Comparative Analysis of Gut Microbiomes in Captive Tigers across *Ex Situ* Facilities in Peninsular Malaysia

M. Gani[a, b], A. R. Mohd-Ridwan[c], N. A. Mohd-Kulaimi[b], M. Khairulmunir[a], M. A. B. Abdul-Latiff[d], and B. M. Md-Zain[a, *]

[a] *Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, 43600 Malaysia*

[b] *National Wildlife Forensic Laboratory (NWFL), Ex-Situ Conservation Division, Department of Wildlife and National Parks (PERHILITAN), KM 10 Jalan Cheras, Kuala Lumpur, 56100 Malaysia*

[c] *Centre for Pre-University Studies, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, 94300 Malaysia*

[d] *Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia, Johor, 84600 Malaysia*

**e-mail: abgbadd1966@yahoo.com*

Received February 10, 2024; revised July 9, 2024; accepted July 10, 2024

Abstract—Tigers residing in captive environments are exposed to novel conditions that have been influenced by human activities, including altered environmental factors, modified diets, and more exposure to humans. This study aimed to identify the gut microbiome communities and diversity of captive tigers, with a primary focus on the Malayan tiger (*Panthera tigris jacksoni*), while considering various tiger subspecies housed across eight different *ex situ* facilities in Peninsular Malaysia. Most Malayan tigers are placed in captivity due to human-tiger conflicts and rescued cases. A total of 65 tiger fecal samples were extracted and then the extracted samples were pooled into 23 genomic DNAs based on locality, followed by age and sex. All samples were analyses by 16S rRNA gene amplicon sequencing targeting the V3–V4 hypervariable region. The result showed that captive tigers had the same gut microbiome composition but different relative abundances of the constituent phyla. Five dominant phyla identified across various *ex situ* facilities were *Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria*, and *Bacteroidota*. The gut microbiome beta diversity was influenced by captivity environment and diet intake of captive tigers. This study also highlighted the presence of potentially pathogenic bacteria, which could significantly impact the health of tigers in captivity. This research provides fundamental information about the gut microbiome of Malayan tigers in captivity to develop the strategies for improving the management of Malayan tiger conservation.

Keywords: metabarcoding, next-generation sequencing (NGS), noninvasive sample, microbial community, *Panthera tigris jacksoni*

DOI: 10.1134/S0026261724604998

Next-generation sequencing (NGS) was known to provide a cost-effective approach to investigating microbiome composition in animals. Most of previous studies used sequencing techniques of polymerase chain reaction (PCR)-amplified 16S rRNA marker by using fecal or gut samples to study the gut microbial composition of wild animals, including big cat groups, Amur tigers, Bengal tigers, leopards, and lions to provide extensive data for studying various factors influencing host-microbiome relationship (Jiang et al., 2020; Mittal et al., 2020; Ning et al., 2020; Sun et al., 2022). The gut microbiome composition varies within or between individuals or a population and might be influenced by the host's genetics, sex, diet, age, environmental factors, and health (Vázquez-Baeza et al., 2016; Jiang et al., 2020; Zhu et al., 2021). Nevertheless, studies on microbiomes in big cats are still rare, and little is known about the felid-microbiome relationship. A limited number of studies has been conducted on wildlife in Malaysia using metagenomic and metabarcoding approaches (Mohd-Yusof et al., 2022; Abdullah-Fauzi et al., 2022; Mohd-Radzi et al., 2022; Osman et al., 2022; Khairulmunir et al., 2023), and there is still a significant gap in knowledge, particularly concerning Malayan tigers.

With only about 200 individuals left in the wild, as reported by the First National Tiger Survey conducted from 2016 to 2020, the Malayan tiger is on the verge of extinction and is listed as one of the critically endangered species in the IUCN Red List of Threatened Species (Kawanishi, 2015). This number does not include Malayan tigers held in captivity. Tigers kept in *ex situ* facilities in Peninsular Malaysia were either involved in human–tiger conflicts or rescued from confiscated cases (PERHILITAN, 2008). Most of these tigers were captured in human–tiger conflict

areas in Peninsular Malaysia and were confirmed to be Malayan tigers. However, certain tigers in captivity in Peninsular Malaysia that were rescued from confiscated cases probably were from subspecies other than the Malayan tiger.

In Peninsular Malaysia, eight *ex situ* facilities where Malayan tigers and other tigers are housed and cared for were identified in this study. These facilities, which include zoos, rehabilitation centers, and breeding centers, are designed to provide controlled environments for the tigers. This study categorized the captive Malayan tigers in Peninsular Malaysia into three types according to the types of captivity environments for Malayan tigers. (1) The National Wildlife Rescue Centre (NWRC) is responsible for rescuing, rehabilitating, and releasing Malayan tigers back into their habitat, following the rewilding concept (Halim et al., 2019). The NWRC has the largest area in Peninsular Malaysia dedicated to the tigers which have been rescued from human-tiger conflict areas, rehabilitated, and kept safe. Other than the NWRC, (2) zoos are one of the enclosure types where captive Malayan tigers live. Zoos in Malaysia are where captive animals are exhibited and used for research, education, training, conservation, and recreation. The Malayan tiger enclosure environment in zoos is smaller than the NWRC environment, and they are exhibited to the public (exhibit yard) more than the tigers kept in the NWRC. (3) A'Famosa Safari Wonderland in the State of Melaka is one of the wildlife safari parks in Malaysia. A few Malayan tigers in A'Famosa Safari Wonderland mostly roam freely in their block. The enclosure and environment arrangements in the safari are slightly different with those in other zoos. This study defined the following three types of tiger captivity conditions: rescue centers, zoos, and safaris. Hence, knowing the different enclosure types for tigers living in captivity might help to understand how the environment and mode of captivity affect the microbiome; the connection to the host's health is also important. Microbiome analyses have great potential to uncover information on the host population, although studies on *Panthera* are still limited (Karmacharya et al., 2019; Mittal et al., 2020).

The goal of the present work was to identify the microbiome composition in eight tiger captive locations across Peninsular Malaysia. Factors potentially influencing the microbiome community in tigers were categorized based on *ex situ* facilities, types of captive environments, dietary intake, age, and sex. Notably, other subspecies such as the Bengal tiger and Indochinese tiger also inhabit *ex situ* facilities in Peninsular Malaysia. These subspecies are kept separate from the Malayan tiger to prevent crossbreeding. Although the primary focus was on the captive Malayan tiger, study encompasses all tigers within *ex situ* facilities, irrespective of their subspecies, to determine how captivity-related factors such as environment type, diet, age, gender and management practices influence the gut microbiome composition of tigers across various *ex situ* facilities in Peninsular Malaysia. In addition, this study attempted to determine the potential pathogenic microbiome present in captive tigers in Peninsular Malaysia. Therefore, this study can provide important information on the relationship between the microbiome of tigers in various captivity environments and diet regime variation, especially for Malayan tigers. It could be one of the methods for understanding the gut microbiome composition of Malayan tigers in captivity and developing the strategies to improve Malayan tiger conservation management. Information obtained could be especially useful for captivity management and rewilding of Malayan tigers in the future.

MATERIALS AND METHODS

Field sampling and fecal collection. A total of 65 tiger fecal samples were collected at eight *ex situ* facilities in Peninsular Malaysia (Fig. 1S). The eight *ex situ* facilities were A'Famosa Safari Wonderland (AF, $n = 15$), Lost World of Tambun (LWOT, $n = 3$), National Wildlife Rescue Center (NWRC, $n = 22$), Zoo Negeri Johor (ZJ, $n = 3$), Zoo Kemaman (ZK, $n = 3$), Zoo Melaka and Night Safari (ZM, $n = 6$), Zoo Negara (ZN, $n = 9$), and Zoo Taiping, Perak (PT-ZT, $n = 4$). At these eight *ex situ* facilities, based on studbook data kept in PERHILITAN and other zoos management, 52 individuals from six facilities were confirmed Malayan tigers (AF, NWRC, ZK, ZM, ZN, ZT), one individual was a Bengal tiger (ZM), and twelve other tigers (NWRC, LWOT, ZJ) probably belonged to the other subspecies. The Bengal tiger and other subspecies were kept in captivity separately from the Malayan tiger subspecies to avoid cross breeding. However, this study was not further investigating at the subspecies level.

The fecal samples from each captive tiger were collected during the morning cleaning at the tiger night den and immediately transferred into a −20°C refrigerator before transporting to the laboratory. All demographical information, such as tiger name, subspecies, age, sex, dietary regimen, body score, and enclosure type, was documented during sampling. While the size of the tiger night den was approximately the same throughout captivity, the environmental enrichment involves implementing activities in captivity that simulate natural behaviors and habitats, promoting the physical and psychological well-being of captive tigers were varied among different *ex situ* facilities. Therefore, the captive environment was categorized into three types on the basis of the type of captivity environment mentioned previously, namely, safari, zoo, and rescue center. The diet provided to tigers in captivity includes chicken meat, beef, lamb meat, and ribs, and some *ex situ* facilities provide live feeding and additional supplement to their tigers. This study categorized dietary intake into three types, as shown in

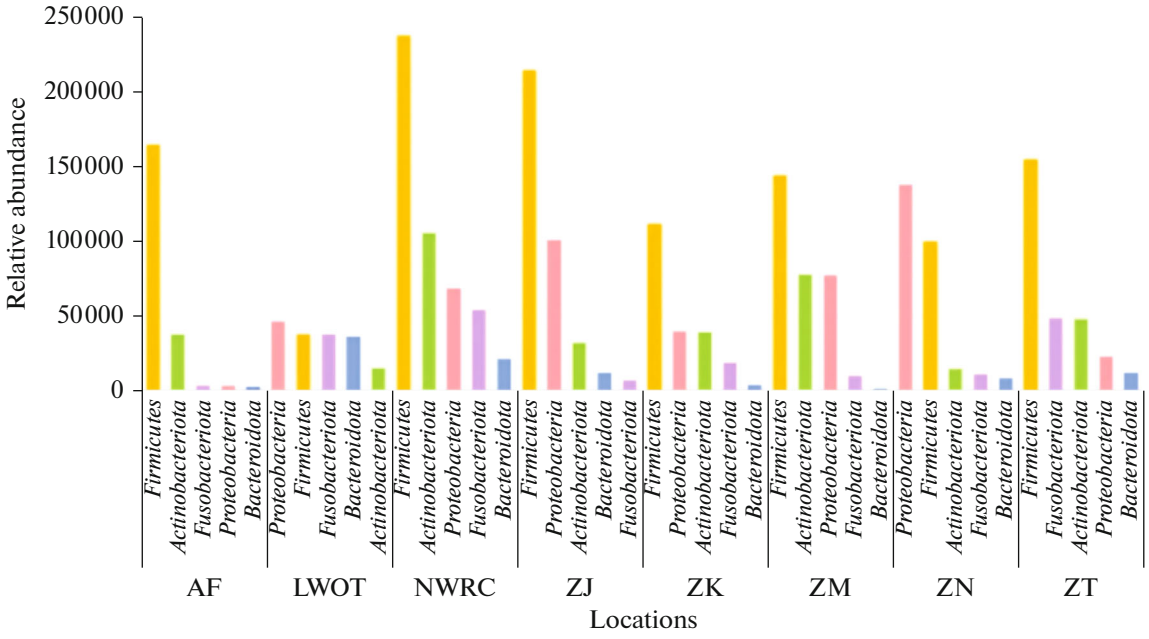

Fig. 1. Relative abundance of the dominant gut microbiome at the phylum level for eight captive tiger locations.

Table 1. In addition to the diet given to all tigers in captivity, most of *ex situ* facilities in Peninsular Malaysia employed a fasting day to their tigers. Therefore, the dietary intake variables were also grouped on the basis of the fasting regimen, which was either one day of fasting per week or nonfasting. Regarding age variables, the age groups were adult tigers more than three years old, subadult tigers 2–3 years old, and juveniles 1–2 years old. PT-ZT-JF and PT-ZT-JM tigers were grouped with the juvenile group because they were turning one year and 2 months old in the year 2021 when the sampling took place. All fecal samples were preserved in different tubes and labeled based on the tiger housename and locality. The samples were transported on dry ice to the laboratory and stored into a −80°C refrigerator until DNA extraction.

DNA extraction and sequencing of 16S rRNA gene amplicons. Genomic DNA samples were extracted from 400 mg of each fecal sample (n = 65) using the innuPREP Stool DNA Kit (Analytic Jena). Fecal samples were obtained from within the fecal mass, rather than from exposed sides with a possibility of contamination. Sixty-five fecal samples of tigers were extracted individually and then pooled into 23 different DNA tubes based on the location of the tigers, followed by their age and sex. The pooled tubes were labeled accordingly, as shown in the first column of Table 2 (e.g., PT-AF-AF: PT stands for *Panthera tigris*, AF represent location, A indicate age, and F denote female sex). Previous studies recommended pooling the extracted DNA samples to save costs and reduce processing time in metabarcoding protocols (Aylagas et al., 2016; Bulcke et al., 2022). Table 2 shows a summary of pooled DNA sample information and category/group.

Purified gDNA was amplified using locus-specific sequence primers of the selected V3–V4 hypervariable region of the 16S rRNA gene with overhang adapters (16S-forward: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 16S-reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). The sequencing libraries were generated using REDiant 2× PCR Master Mix (1st BASE). Dual indices were attached to the amplicon PCR using the Illumina Nextera XT Index Kit version 2 according to the manufacturer's protocols. The library was normalized and pooled according to the protocol recommended

Table 1. Dietary regimen given to tigers in captivity in a week

Diet regime (1 week)	Category 1 (DC1)	Category 2 (DC2)	Category 3 (DC3)
Frozen meat (chicken and beef)	✓	✓	✓
Live meal/Bone/fresh meat		✓	✓
Additional supplement			✓

Table 2. Sample information of captive tigers in Peninsular Malaysia

Pooled DNA ID	Facility locations	No. of samples	Environment types	Diet categories	Fasting regime
PT-AF-AF PT-AF-AM	AF	15	Safari	DC2	Fasting
PT-NWRC-AF PT-NWRC-AM PT-NWRC-JF PT-NWRC-JM PT-NWRC-SAF	NWRC	22	Rescue Center	DC3	Fasting
PT-LWOT-AF PT-LWOT-AM	LWOT	3	Zoo	DC3	Fasting
PT-ZJ-AF PT-ZJ-AM PT-ZJ-JF	ZJ	3	Zoo	DC1	Fasting
PT-ZK-AF PT-ZK-AM	ZK	3	Zoo	DC3	Fasting
PT-ZM-AF PT-ZM-AFWB PT-ZM-AM	ZM	6	Zoo	DC3	Fasting
PT-ZN-AF PT-ZN-AM PT-ZN-JM	ZN	9	Zoo	DC1	Non-fasting
PT-ZT-AF PT-ZT-JF PT-ZT-JM	ZT	4	Zoo	DC2	Non-fasting

by Illumina and proceeded to 300-paired end sequencing using the MiSeq platform.

Sequence processing and 16S microbial data analysis. Raw sequencing data were trimmed using Cutadapt 3.5 (Martin, 2011) to remove any remaining Illumina adapters, primers, and bases below average quality. The quality assessment of sequencing reads was conducted using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then, the trimmed sequences were merged to create a single consensus sequence for each pair using the DADA2 pipeline (Callahan et al., 2016). Chimeric sequences were screened and taxonomy assignment was done using the SILVA nr database V138.1. Clustering reads into amplicon sequence variants (ASV) were generated using the DADA2 pipeline.

The microbial community data were analyzed using Rstudio version 4.3.2. The reads were rarefied using the Rarefy package (Thouverai et al., 2021) to compare microbiomes on an equal basis to take into account the different sequencing depths when comparing alpha and beta diversities. Alpha diversity analyses were evaluated within the sample using two indices, which are Chao1 and Shannon, using the phyloseq (McMurdie and Holmes, 2013) and ggplot2 (Wickham, 2016) packages in Rstudio. The indices were estimated to indicate species richness, abundance, and evenness within captive tiger populations. Significance was calculated using the Mann–Whitney U test ($p < 0.05$) to observe intergrouping variability. Meanwhile, beta diversity metrics was displayed in principal coordinates analysis (PCoA) plots and were calculated based on the weighted Unifrac and Bray–Curtis distance method to determine the variation in communities and structure of the gut microbiome among groups of tigers in captivity. Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis2 function in vegan packages (Oksanen et al., 2016) to determine the significance of the differences in the gut microbiome com-

munity composition among defined groupings with 999 permutations based on Bray–Curtis distance.

RESULTS

Bacterial 16S rRNA sequence data assessment and general microbiome composition. High-throughput sequencing of 23 pooled gDNA from 65 gDNA fecal samples generated 4064357 raw reads. After quality filtering procedures, 2345165 clean reads were used in the microbial community analysis. The sequences per sample ranged between 75206 and 177175. A total of 1555 ASVs were generated, encompassing identified sequences of 17 phyla, 24 classes, 66 orders, 120 families, 303 genera, and 154 species. All sequences data from 23 pooled DNA were submitted to GenBank with BioProject accession number PRJNA901222. Overall, five major dominant phyla were found in all captive tigers: *Firmicutes* (49.76%), *Proteobacteria* (21.31%), *Actinobacteria* (15.91%), *Fusobacteria* (8.28%), and *Bacteroidota* (4.31%). *Firmicutes* were highly abundant in the gut microbiome in six *ex situ* facilities, namely, AF (76.89%), NWRC (48.60%), ZJ (58.31%), ZK (51.86%), ZM (46.18%), and ZT (53.74%). Meanwhile, *Proteobacteria* had the highest abundance in the groups from LWOT and ZN, with 25.68 and 50.31%, respectively (Fig. 1). At the genus level, *Collinsella* (14.1%) was the most frequently detected in all captive tiger samples followed by *Paeniclostridium* (11.02%), *Escherichia–Shigella* (10.54%), *Clostridium sensu stricto 1* (9.81%), and *Fusobacterium* (7.02%). Among ten dominant genera in all captive tigers found in this study, *Paeniclostridium*, *Escherichia–Shigella*, *Clostridium sensu stricto 1*, *Fusobacterium*, *Peptoclostridium*, *Solobacterium*, and *Ignatzschineria* were known to cause infections in the digestive systems of humans and animals, including species of wildlife, by previous studies (Zhang et al., 2012; Honneffer et al., 2014; Yu et al., 2015; Brennan and Garret, 2019; Yang et al., 2019; Kalender et al., 2023).

According to their relative abundance, the bacterial communities in all captive tigers belonging to all groups were similar at the phylum level but differed in genus composition (Fig. 2). In general, all five main groups contained the same dominant phyla of *Firmicutes, Proteobacteria, Actinobacteriota, Fusobacteriota*, and *Bacteroidota* (Figs. 2a–2e). Among the environment types of captivity groups, tigers in safari groups had *Paenisclostridium* (38.2%) as the most abundant genus, followed by *Collinsella* (17.25%). Meanwhile, *Collinsella* was found to have the highest abundance in the microbiome of captive tigers inhabiting rescue centers (19.05%) and zoos (12.21%), followed by *Escherichia–Shigella* (rescue centers, 10.57%; zoos, 11.72%). *Ignatzschineria* was among 10 genera with the highest relative abundance in the zoo groups, but was not found in the safari and rescue center groups. The diet categories of the captive tigers showed a different relative abundance of microbiome composition at the genus level (Fig. 2g). DC1 had a higher abundance of the genera *Acinetobacter*, *Comamonas*, and *Providencia* compared with DC2 and DC3. According to the relative abundance in the fasting regimen, *Collinsella* had the highest abundance in the 1-day fasting group, whereas *Fusobacterium* had the highest abundance in the nonfasting group (Fig. 2h).

Among the age groups, the phyla *Chloroflexi* and *Desulfobacterota* were absent in juvenile tigers but present in adults and subadults. Meanwhile, *Dependentiae, Gemmatimonadota, Myxococcota*, and *Verrucomicrobiota* were absent in the bacterial community of the subadult and juvenile groups but were present only in the adult group. At the genus level, the dominant genera were *Collinsella*, *Paeniclostridium*, *Clostridium sensu stricto 1*, and *Escherichia–Shigella* in adult and juvenile tigers, whereas the genera *Cetobacterium* and *Peptoclostridium* had the highest abundance in subadults. However, according to sex group, the genera *Megamonas* and *Peptostreptococcus* had higher abundance in male tigers, whereas the genus *Ignatzschineria* was dominant in female tigers than in male tigers.

Alpha and beta diversities of the gut microbiome between study groups. To compare the alpha and beta diversities, the reads were rarefied at a rarefaction depth of 75206 reads to compare the microbiomes on an equal basis. To understand whether the environment of captivity and the diet given to captive tigers play a role in shaping the gut microbiome, alpha and beta analyses were conducted for the environment types, diet category, and fasting regimen defined in this study. Additional comparison analyses were performed in age and sex groups to observe whether gut microbiome composition affected these variables. Alpha diversity analysis showed there were no significant differences observed in community richness for Chao1 and Shannon indices in all groups ($p > 0.05$, Mann–Whitney U test).

Beta diversity showed a pattern of separation between different groups and revealed significant differences in microbiome diversity depending on locations, environment types, and diet category groups ($p < 0.05$, PERMANOVA; Figs. 3a–3c). The scattered point described how the samples within each variable group were dispersed and the closer the point was, the more similar was the gut microbiome composition. PERMANOVA analyses results varied significantly between captive tigers in NWRC and ZJ ($p = 0.039$, PERMANOVA), NWRC and ZN ($p = 0.022$, PERMANOVA), and NWRC and LWOT ($p = 0.044$, PERMANOVA). R^2 indicates the distance variance of the groups in the analysis. Moreover, significant differences were found between the safari and rescue center and also between safari and zoo groups ($p = 0.044$ and 0.047, respectively; Fig. 3b). A separate cluster was displayed among diet categories in the

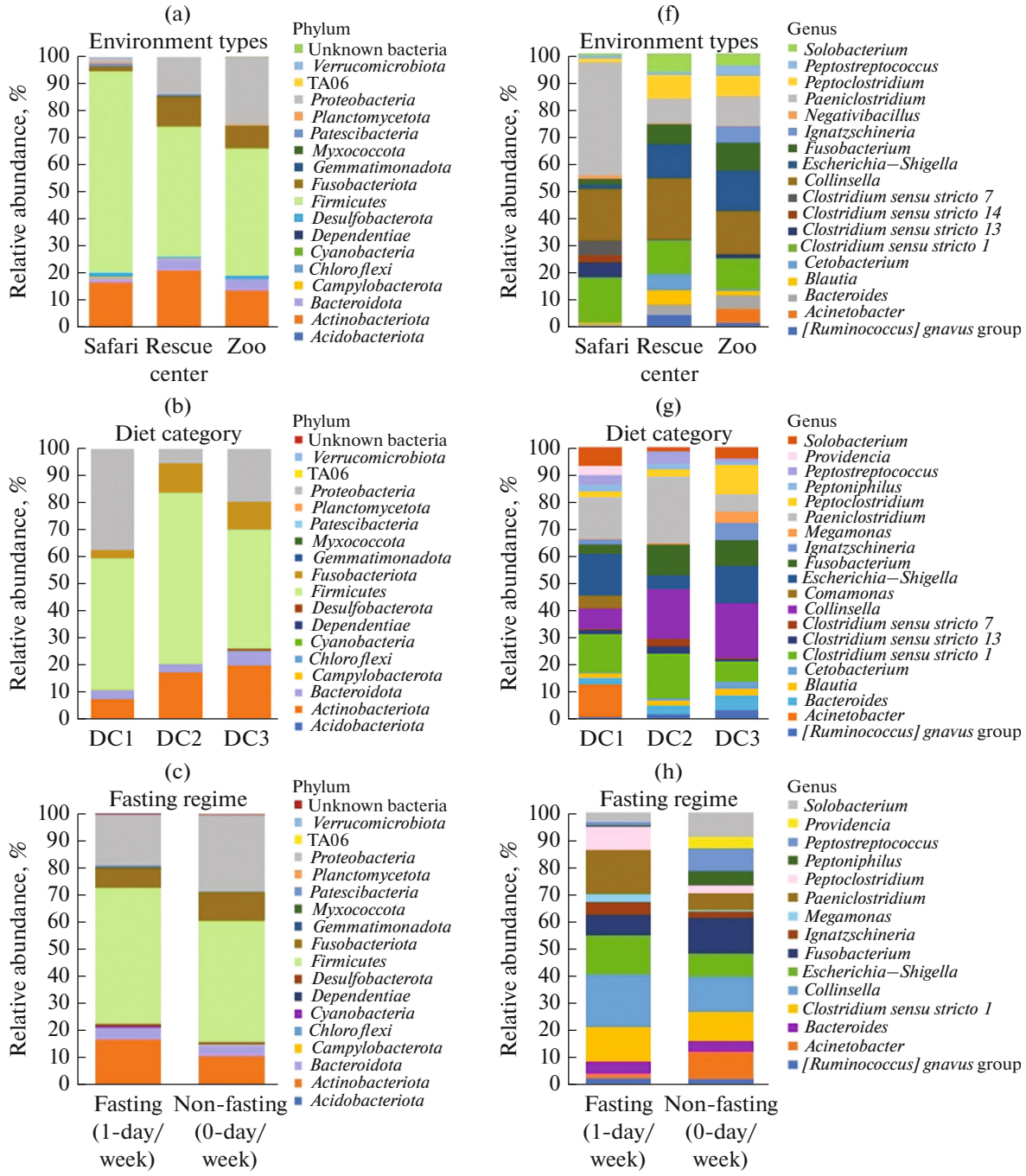

Fig. 2. Relative abundance of microbiome composition among groups of captive tigers at the phylum (a–e) and genus (f–j) levels.

PCoA plot (Fig. 3c) and revealed a significant difference between DC1 and DC3 (p = 0.032, PERMANOVA). Meanwhile, fasting regimen, age and sex groups showed no grouping based on the PCoA plot analysis and there was no significant difference observed between these groupings based on PERMANOVA analyses and this was supported with Mann–Whitney U test based on Chao1 and Shannon indices.

DISCUSSION

Microbiome composition of captive tigers and potential pathogenic bacteria. Overall, in the microbiome composition of captive tigers in Peninsular Malaysia, the phyla *Firmicutes* and *Proteobacteria* were found to be generally dominant in the gut of members of the genus *Panthera*, which was in agreement with the results of previous studies (Karmacharya et al., 2019; Jiang et al., 2020; Ning et al., 2020; Mittal et al.,

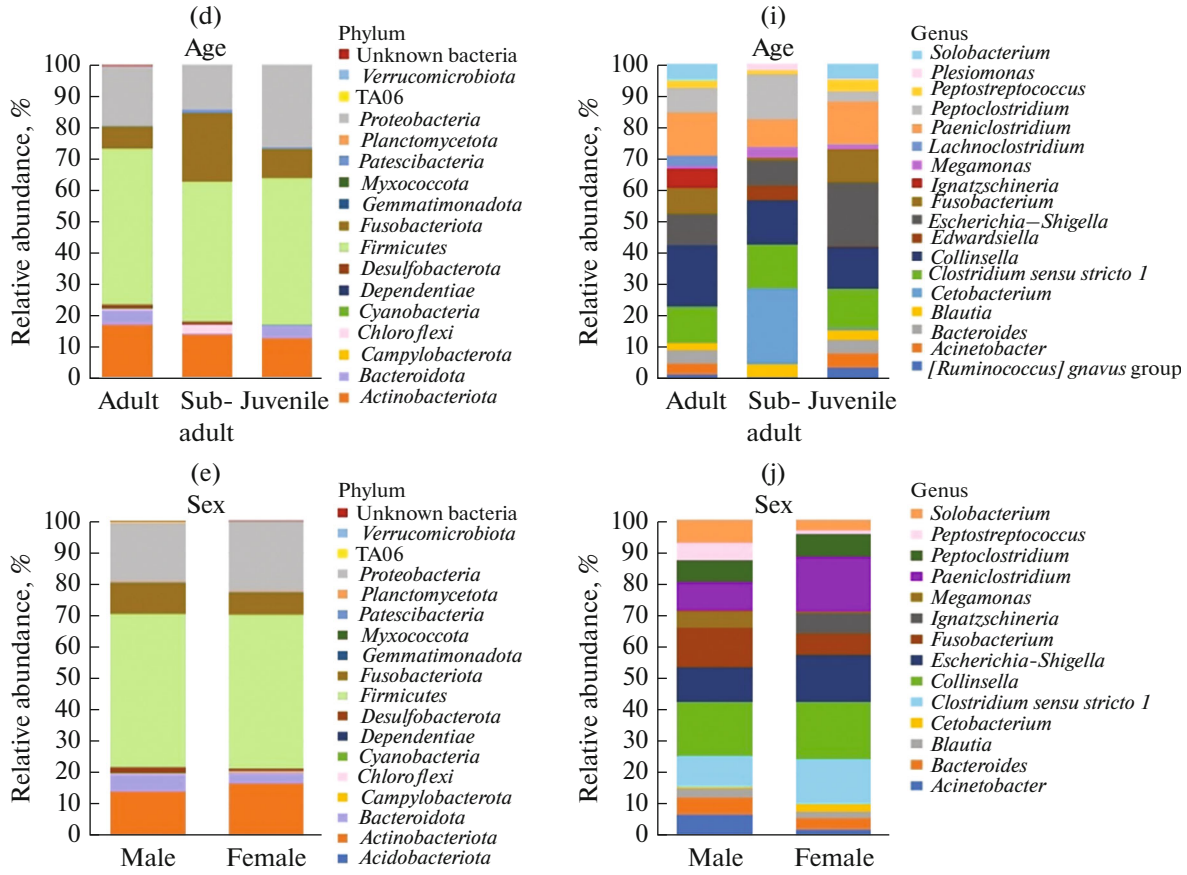

Fig. 2. (Contd.)

2020; Sun et al., 2022; Khairulmunir et al., 2023). In the microbiome composition of captive tigers, *Firmicutes* and *Proteobacteria* were predominant in all variables groups defined in this study. However, the composition at the genus level differed depending on variables groups. The gut microbiome composition serves as an indicator of bacterial responses to the environment and also provides insights into the presence of disease (Bahrndorff et al., 2016; de Jonge et al., 2022). Notably, the abundance of the genus *Lactobacillus* was found higher in the tigers at ZT than in those in NWRC and ZJ, while this genus was not found in the remaining group. The results may be attributed to the weaning off process of PT-ZT-JF and PT-ZJ-JM from their mother (PT-ZT-AF) at the time of fecal sampling, which occurred when these individuals were one year and 2 months old. Furthermore, the tiger keepers observed the behavior where the mother would consume leftovers from her offspring, allowing her offspring to eat first and consume any remaining portions. This behavior likely resulted in a lack of the *Bacteroides* genus in the mother, while the offspring showed an increased presence of *Bacteroides* in their gut microbiome, as they had more access to food and nutrients. *Fusobacterium* also found higher in PT-ZT-JF and PT-ZJ-JM but lower in PT-ZT-AF.

This study provides baseline data by underlining the presence of enteric and opportunistic pathogenic fecal bacteria in captive tigers, offering insights to improve the captivity management and husbandry practices for Malayan tigers in the Peninsular Malaysia. Changes in the microbiome composition can confer resistance to or promote infection by pathogenic bacteria and will cause an imbalance called dysbiosis which leads to infection and can trigger several diseases (Rolhion and Chassaing, 2016). Our finding discovered a high abundance of *Clostridium sensu stricto 1* (*perfringens*), found in 14 pooled samples from six locations (AF, NWRC, ZJ, ZK, ZM, and ZT). *Clostridium perfringens* belonging to the phylum Firmicutes is considered the most important cause of clostridial enteric disease. Highly virulent *C. perfringens* type A had been identified in a Siberian tiger and in a lion with hemorrhagic enterocolitis (Zhang et al., 2012). *Clostridium* and *Blautia* were also associated with parasitic infections (Ning et al., 2020). In addition, high relative abundance of *Peptoclostridium* (NWRC, ZK, and ZM) and *Paeniclostrodium* (AF and ZJ) was detected in the phylum *Firmicutes*. These two genera have been reported as major pathogens associated with infectious diarrhea and enterocolitis in both

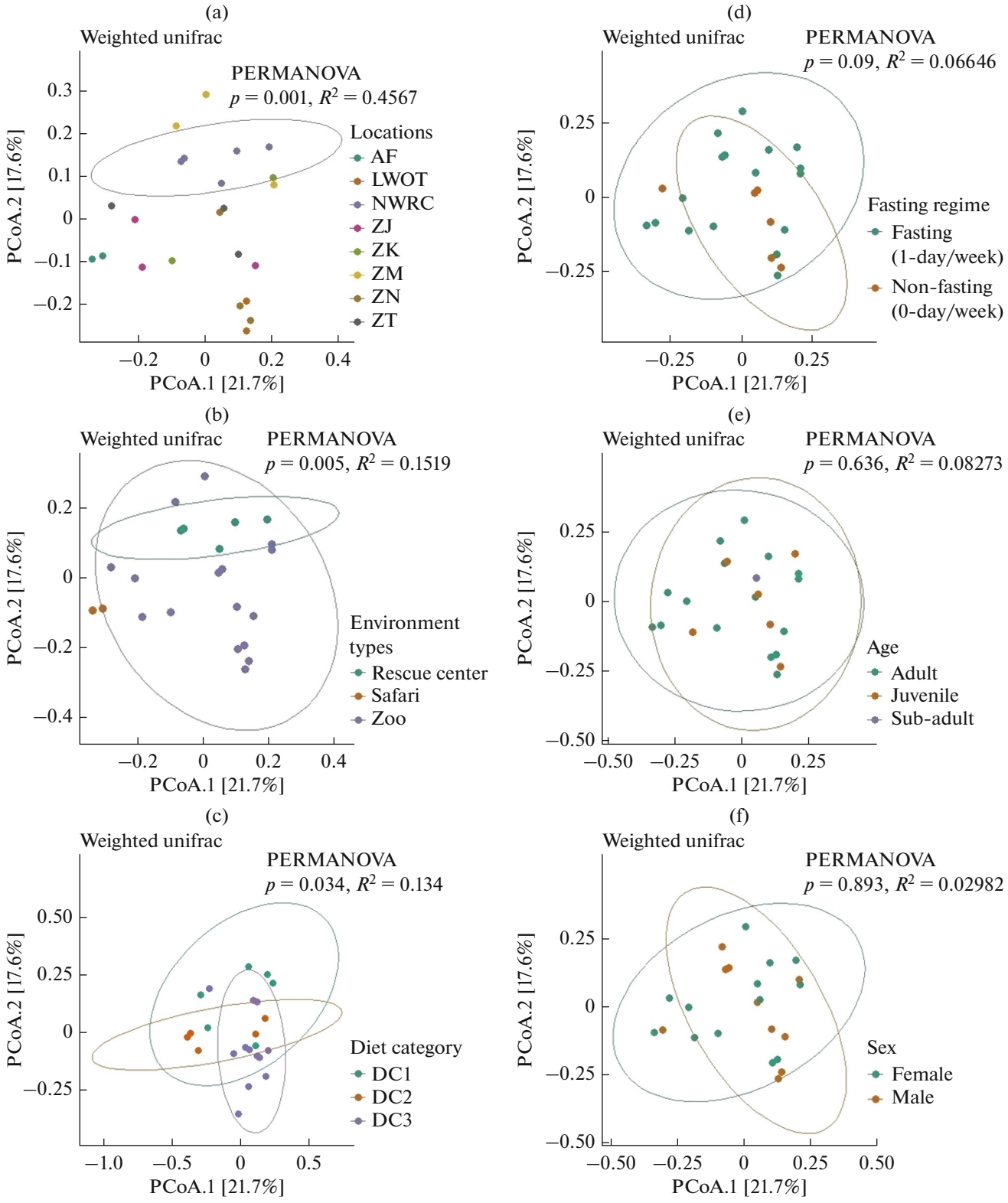

Fig. 3. Principal coordinate analyses (PCoA) of the gut microbiome based on the weighted Unifrac and Bray–Curtis distance matrix for all groups designated in this study. The *p*-values and variations of the PERMANOVA tests are noted at the top of each PCoA plot.

humans and animals (Luo et al., 2016; Nyaoke et al., 2020; Weese, 2020).

According to Brennan and Garret (2019), *Fusobacterium* was also considered an opportunistic pathogen due to the higher prevalence of this genus associated with various diseases in humans and animals, including predental abscess, IBD, carcinoma, and colorectal cancer clinical samples. The gut microbiome of tigers at LWOT and Malayan tigers at ZT was dominated by *Fusobacterium* (20.78 and 16.14%, respectively) compared to the tigers in other *ex situ* facilities (1.72–6.23%). Meanwhile, the *Escherichia–Shigella* group was discovered as the most prevalent genus of the phylum *Proteobacteria*, especially prevalent in LWOT, NWRC, ZJ, and ZK. A previous study showed *Escherichia–Shigella* to be gastrointestinal pathogens (Moon et al., 2018) and the *Proteobacteria* proportion (increase in *Proteobacteria*, family *Enterobacteriaceae* and decrease in the phylum *Firmicutes*, genera *Faecalibacterium* and *Blautia*) was related to IBD (Suchodolski et al., 2015; Vázquez-Baeza et al., 2016). Meanwhile among the *Actinobacteria* phylum, the primary representatives, *Collinsella* and *Slackia*, were present in all captive tigers. Handl et al. (2011) found *Collinsella* to be a prominent taxon in dogs, meanwhile *Eggerthella* and *Olsenella* were common in cats. Notably, Suchodolski et al. (2015) found a significant increase in *Streptococcus* and *Collinsella* in diarrheic cats. Occasionally, the increased abundance of *Collinsella* driven by fiber consumption is minimal in the *Panthera* group (Mittal et al., 2020; Ning et al., 2020). Most tigers in this study exhibited good health during field sampling. Therefore, the findings of potentially harmful bacteria can be used as baseline data to help in monitoring of captive tigers to improve their overall health in future.

Microbiome diversity shaped by *ex situ* facility environments and dietary intake differences. The gut microbiome of captive tigers in Peninsular Malaysia exhibited distinctive differences in composition influenced by both environmental factors and dietary patterns. This study indicated a marked variation in beta diversity analysis using PCoA weighted Unifrac and PERMANOVA among these captive populations. This supported the previous studies that found significant changes in the tiger dietary habit and environment differences (Jiang et al., 2020; Ning et al., 2020; Zhu et al., 2021). Environmental factors, such as habitat conditions and captive enclosures, play a role in shaping the microbial diversity by influencing the exposure to diverse microbial communities. Significant differences were observed in beta diversity analyses between NWRC and AF, ZJ, and ZN. The difference between NWRC and the other three locations may be attributable to the built-in environmental (enclosure) factors, such as size, and to the differences in such environmental enrichment activities as cognitive, social, and sensory ones, as well as to feeding enrichment, including the diet given to captive tigers.

In addition, dietary composition, including meal types and additional diet content, significantly affects the gut microbiota composition, leading to observable differences in beta diversity among tiger populations. This study showed that the diet DC1 (ZN and ZJ) with DC3 (NWRC, LWOT, ZK and ZM) resulted in a significantly different gut microbiome diversity. The DC3 groups were given a different prey species through live feeding, and additional diet included raw eggs, red palm oil, or cod liver oil in chicken or beef meat. The abundance of *Fusobacterium* and *Bacteroides* showed a tendency to increase with different diet types, suggesting that a wider range of diet intakes leads to higher abundance of these genera. Moreover, the genus *Fusobacterium* was found to be the most dominant in nonfasting groups, consistent with the findings of Khairulmunir et al. (2023), whereas they found *Fusobacterium* was one of the most dominant genera in the normal phase (not fasting day). Jiang et al. (2020) also revealed a higher abundance of *Fusobacterium* in the diet group of meat-feed tigers compared to mixed- and milk-fed tigers, supporting the hypothesis that high *Fusobacterium* was associated with meat digestion. According to Ley et al. (2008), the role of *Fusobacterium* activity is to break down proteins to obtain such growth substrates as amino acids and peptides, particularly in carnivores. Hence, the higher abundance in *Fusobacterium* in nonfasting group may contribute by this activity as the group were given meat every day compared to the *ex situ* facilities that employed fasting day to their captive tigers.

The genera *Bacteroides* and *Blautia* were observed increased in diet category in this study. *Bacteroides* was discovered in a person who had consumed high protein and fat (Amato et al., 2015). Durand et al. (2017) found that elevated levels of *Bacteroides* and *Blautia* in humans were linked to good nutrition. Our study observed an increase in *Blautia* and *Bacteroides* across each diet category from DC1 to DC3, consistent with the finding by Ning et al. (2020) in captive settings. Therefore, the increased abundance of *Bacteroides* and *Blautia* in diet category groups DC2 and DC3, compared to the DC1 group, indicates supplements and fresh meat probably provided good nutrition to captive tigers in Peninsular Malaysia. Raw eggs and palm oil are considered to be components of a high-fat diet. A high-fat diet can increase abundance of beneficial bacteria, but some fats are linked to obesity-promoting changes in microbiomes (Hildebrandt et al., 2009; Zhang, 2022). Obesity is associated with a high *Firmicutes* and low *Bacteroidota* ratio (Magne et al., 2020); according to the data on body scores obtained during fecal sampling, the tigers' body score was 3 to 4, indicating some of the tigers were more prone to obesity. However, this data is only a baseline that can be used to assess the tiger condition in captivity. Additional investigation needs to be carried out.

In conclusion, this study utilized fecal sample metabarcoding and 16S rRNA amplicon sequencing

to characterize microbiome diversity and composition in captive tigers. The findings of this study indicated that environmental factors and dietary intake among captive tiger groups lead to differences in the gut microbiome composition. Furthermore, the potentially pathogenic microbiome revealed in our study may probably present a risk to the health and husbandry of the captive Malayan tiger. These results can be used as one of the strategies for understanding the interaction between tigers and its microbiome in captivity especially in Malayan tiger to further develop strategies to improve the management of Malayan tiger conservation, particularly in captivity, and possible use for future rewilding efforts.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S0026261724604998.

ACKNOWLEDGMENTS

Special thanks to the Malaysian Tiger Genotyping System (MyTAG) teams (Frankie Thomas Sitam, Dr Zubaidah Kamarudin, Dr Siti Suzana Selamat, Nik Mohd Zamani Awang, Norfadhilah Ahmad Subaker, Mohammad Zafrin Mohd Zamri, Khairul Anwar Mislan, Muhammad Fauzan Muhamad Zuber, Hartini Ithnin and Norsyamimi Rosli), staffs and tigers' keepers at NWRC, ZM, ZT, ZN, ZK, ZJ, LWOT and AF in helping sample and data collections. The authors acknowledge the Department of Wildlife and National Parks (PERHILITAN) Peninsular Malaysia for the support and permission to conduct this study (Permit no. P06/10/2020). We thankful to the Universiti Kebangsaan Malaysia for the ethical permit approval in our research protocol (UKMAEC approval number: FST/2021/BADRUL MUNIR/22-SEPT./1198-OCT.-2021-OCT.-2023-NAR-CAT2).

AUTHOR CONTRIBUTION

All authors were involved in the study conception and design. MG, NAMK, KM conducted field sampling. MG conducted laboratory work and data analyses. MG wrote the manuscript. ARMR, MABAL, BMMZ critically revised the intellectual content. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by Fundamental Research Grant Scheme (FRGS), FRGS/1/2020/WAB11/UKM/01/1 funded by the Ministry of Higher Education (MOHE), Malaysia and the part for this research is supported by Malaysia Government of Malaysia under the 12th Malaysia Plan Project: Strengthening Wildlife Forensics, *Ex-Situ* Conservation and Biobanking-Phase 2 (Project Code: P23071000810008), and also funded by the Geran Universiti Penyelidikan, GUP-2022-043 Universiti Kebangsaan Malaysia.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Wildlife Permit No.: P06/10/2020 -Ethical Permit: UKMAEC approval number: FST/2021/BADRUL MUNIR/22-SEPT./1198-OCT.-2021-OCT.-2023-NAR-CAT2).

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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