GUT MICROBIOME AND EPIGENETICS ANALYSIS OF COMMON LONG-TAILED MACAQUE *Macaca fascicularis fascicularis* AND BURMESE LONG-TAILED MACAQUE *M. fascicularis aurea* IN DIFFERENT HABITAT TYPES

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Zoology
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การวิเคราะห์ไมโครไบโอมและอีพีเจเนติกส์ของลิงหางยาวชนิดย่อยธรรมดา *Macaca fascicularis fascicularis* และลิงหางยาวชนิดย่อยพม่า *M. fascicularis aurea* ในถิ่นที่อยู่ที่ต่างกัน

นายราซา มูฮัมหมัด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ราชา มูฮัมหมัด : การวิเคราะห์ไมโครไบโอมและอีพีเจเนติกส์ของลิงหางยาวชนิดย่อยธรรมดา *Macaca fascicularis fascicularis* และลิงหางยาวชนิดย่อยพม่า *M. fascicularis aurea* ในถิ่นที่อยู่ที่ต่างกัน. (GUT MICROBIOME AND EPIGENETICS ANALYSIS OF COMMON LONG-TAILED MACAQUE *Macaca fascicularis fascicularis* AND BURMESE LONG-TAILED MACAQUE *M. fascicularis aurea* IN DIFFERENT HABITAT TYPES) อ.ที่ปรึกษาหลัก : ศ. ดร.สุจินดา มาลัยวิจิตรนนท์, อ.ที่ปรึกษาร่วม : ศ. ดร.สัญชัย พยุงกร

Macaca fascicularis หรือลิงหางยาว มีการแพร่กระจายอย่างกว้างขวางทั่วทั้งเอเชียตะวันออกเฉียงใต้ โดยแบ่งออกเป็น 10 ชนิดย่อย ซึ่งใน 10 ชนิดย่อยนี้ *M. f. fascicularis* (*Mff*) มีการแพร่กระจายกว้างขวางที่สุด ส่วน *M. f. aurea* (*Mfa*) จัดเป็นลิงโลกเก่าเพียงชนิดเดียวที่มีรายงานการใช้เครื่องมือหินในการหาอาหาร ในการศึกษาก่อนหน้านี้พบว่า *Mff* และ *Mfa* มีลักษณะทางพันธุกรรมที่แตกต่างกัน และส่วนใหญ่ *Mfa* อาศัยอยู่บริเวณชายฝั่งหรือบริเวณปากแม่น้ำมากกว่า *Mff* ดังนั้นคาดว่าสภาพแวดล้อมน่าจะมีส่วนสำคัญในการคัดเลือกตามธรรมชาติในพฤติกรรมการใช้เครื่องมือหิน ในปัจจุบันนี้พบว่ามีความสนใจศึกษาวิจัยเกี่ยวกับความสัมพันธ์ระหว่างจุลินทรีย์ในลำไส้และการพัฒนาของสมอง หรือ gut-brain axis ดังนั้นโครงการวิจัยนี้จึงสนใจศึกษาผลของลักษณะทางพันธุกรรมของเจ้าบ้าน (*Mff* และ *Mfa*) ถิ่นอาศัย (ป่าชายเลนและเกาะ) และอาหาร (อาหารที่ได้รับจากมนุษย์และอาหารจากธรรมชาติ) ต่อองค์ประกอบของจุลินทรีย์ในลำไส้ โดยทำการคัดเลือก *Mff* และ *Mfa* มาอย่างละ 2 ประชากร ที่อาศัยอยู่บนเกาะและอยู่ในป่าชายเลน ตามลำดับ นอกเหนือจากอาหารตามธรรมชาติแล้วพบว่าในระหว่างการสำรวจ *Mff* ยังได้รับอาหารเพิ่มเติมจากมนุษย์อีกด้วย จากการเก็บอุจจาระที่ลิงถ่ายออกมา (ประชากรละ 30 ตัว) นำมาวิเคราะห์จุลินทรีย์ในลำไส้ด้วยการศึกษาลำดับของยีนส่วน 16S rRNA โดยใช้เทคโนโลยี Oxford Nanopore ผลการศึกษาพบว่าประชากร *Mff* มีความหลากหลายของแบคทีเรีย (alpha diversity) มากกว่า *Mfa* ที่มีถิ่นอาศัยชนิดเดียวกัน โดยไฟลัมเด่นของจุลินทรีย์ในลำไส้ของลิงทั้งสองชนิด คือ Firmicutes และ Bacteroidetes โดย *Mfa* มีค่าปริมาณเชิงสัมพัทธ์สูงกว่า *Mff* อย่างมีนัยสำคัญทางสถิติ ซึ่งบ่งชี้ให้เห็นว่าองค์ประกอบของจุลินทรีย์ในลำไส้มีความแปรผันขึ้นกับชนิดของอาหารเป็นหลัก แต่ปัจจัยจากพันธุกรรมของเจ้าบ้านและถิ่นอาศัยก็ยังคงมีส่วน จากการเก็บอุจจาระลิงในช่วงที่รัฐบาลออกมาตรการจำกัดการเดินทาง (lockdown) เนื่องจากการแพร่ระบาดของโรคโควิด-19 ระหว่างวันที่ 12 กรกฎาคม ถึง 6 สิงหาคม 2565 ลิงในธรรมชาติจึงได้รับอาหารจากมนุษย์ลดลง ทำให้ *Mff* ที่อาศัยอยู่บนเกาะเปิด เผชิญกับสภาวะขาดแคลนอาหาร และได้พัฒนาพฤติกรรมการใช้เครื่องมือหินแบบ “pound-hammering-like” ในการหาอาหาร เช่น หอยนางรม ซึ่งมีประสิทธิภาพต่ำกว่า “pound-hammering” ที่มีรายงานมาก่อนหน้านี้ใน *Mfa* และลิงลูกผสม *Mfa* x *Mff* ซึ่งสะท้อนให้เห็นว่าพันธุกรรมของ *Mfa* มีผลต่อทักษะการใช้เครื่องมือหินในลิงหางยาว โดยพบว่าลิงบนเกาะเปิดส่วนใหญ่ที่แสดงพฤติกรรมนี้อยู่ในระยะโตเต็มวัยสมบูรณ์และระยะโตเต็มวัย โดยร้อยละ 88 เป็นลิงเพศผู้ ทั้งนี้อาจเนื่องมาจากหินที่ใช้เป็นเครื่องมือมีน้ำหนักมาก ดังนั้นเพื่อให้เข้าใจถึงความสัมพันธ์ของ gut-brain axis และพฤติกรรมการใช้เครื่องมือหิน จึงได้เก็บตัวอย่างพลาสม่าจากลิงหางยาวที่อาศัยอยู่ในป่าชายเลน จำนวน 2 ประชากร โดยประชากรแรกเป็นลิงชนิด *Mfa* ที่สามารถใช้เครื่องมือหินได้ ส่วนอีกประชากรเป็นลิงชนิด *Mff* ที่ไม่สามารถใช้เครื่องมือหินได้ นำมาตรวจวัดระดับทริปโตเฟน (Trp) และซีโรโทนิน (5-HT) ในพลาสม่าด้วยเทคนิค HPLC แต่ด้วย 5-HT มีระดับที่ต่ำมากในพลาสม่าจึงไม่สามารถตรวจวัดได้ด้วยเทคนิค HPLC ดังนั้นในการศึกษาครั้งนี้จึงเปรียบเทียบเฉพาะระดับ Trp จากการแบ่งลิงในแต่ละประชากรออกเป็น 3 กลุ่มตามช่วงวัย คือ ระยะโตเต็มวัยสมบูรณ์ ระยะโตเต็มวัยและระยะเด็ก พบว่าระดับ Trp ในพลาสม่าไม่มีความแตกต่างกันระหว่างช่วงวัยทั้งในลิงประชากรเดียวกันและระหว่างประชากร ยกเว้นลิง *Mfa* ในระยะโตเต็มวัยสมบูรณ์ที่มีระดับ Trp ในพลาสม่าสูงกว่าลิง *Mff* ระยะโตเต็มวัยสมบูรณ์อย่างสัมพันธ์กับพฤติกรรมการใช้เครื่องมือหินที่พบได้มากกว่า ผลจากการศึกษานี้แสดงให้เห็นถึงความสัมพันธ์ระหว่างจุลินทรีย์ในลำไส้ การปรับอาหาร และพฤติกรรมทางวัฒนธรรม โดยเฉพาะอย่างยิ่งการใช้เครื่องมือหินในลิงมะแคดเหล่านี้ ที่นำไปสู่ความรู้เกี่ยวกับปฏิสัมพันธ์ที่ซับซ้อนระหว่างพันธุกรรมของเจ้าบ้าน อาหารและ จุลินทรีย์ในลำไส้

สาขาวิชา	สัตววิทยา	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Raza Muhammad : GUT MICROBIOME AND EPIGENETICS ANALYSIS OF COMMON LONG-TAILED MACAQUE *Macaca fascicularis fascicularis* AND BURMESE LONG-TAILED MACAQUE *M. fascicularis aurea* IN DIFFERENT HABITAT TYPES. Advisor: Prof. SUCHINDA MALAIVIJITNOND, Ph.D. Co-advisor: Prof. SUNCHAI PAYUNGPORN, Ph.D.

Macaca fascicularis (long-tailed macaques) occupy a wide habitat range in Southeast Asia and are divided into 10 subspecies. Among the 10 subspecies, *M. f. fascicularis* (*Mff*) encompassed the largest distribution, and *M. f. aurea* (*Mfa*) was the only Old-World monkey reported using stone as tools to forage encased food. Previous studies indicated the distinctive genetic characteristics between the two subspecies, and *Mfa* primarily inhabited coastal and estuary habitats to a greater extent than the overall *Mff* populations. Thus, the ecological conditions appeared to be conducive to natural selection of stone-tool use behavior. Based on the current interest in an association between gut microbiota and brain development, namely the gut-brain axis, this study aimed to investigate the effect of host genetics (*Mff* and *Mfa*), habitat types (mangrove and island) and diets (human-fed foods or natural foods) on gut microbiota composition. Two populations, each of *Mff* and *Mfa*, residing on the island and in mangrove forests were recruited. In addition to their natural foods, only *Mff* could access to human-fed foods during the field survey. Fecal specimens (n = 30 for each population) were collected for gut microbiota analysis using 16S rRNA gene sequencing on Oxford Nanopore Technologies. *Mff* populations exhibited higher bacterial species richness (alpha diversity) in their gut microbiota compared to respective *Mfa* populations living in the same habitat types. The dominant bacterial phyla in the gut microbiota of both subspecies were Firmicutes and Bacteroidetes; however, *Mfa* exhibited a significantly higher relative abundance of these phyla compared to the *Mff*. This denoted that the composition of gut microbiota primarily differed based on variations in diet, although the influence of host genetics and habitat type should not be disregarded. Since the fecal specimen collections were performed during the COVID-19 lockdown (12 July – 6 August 2022) when the human-fed foods were diminished, an island-living, namely Koh Ped, *Mff* faced food scarcity. They developed stone-tool use "pound-hammering-like" behavior to forage for natural foods, i.e., oysters, which was less proficient than the pound-hammering behaviors previously reported in the *Mfa* and *Mfa* x *Mff* hybrids, suggesting a potential genetic contribution of *Mfa* to the skill of stone-tool manipulation in *M. fascicularis*. Koh Ped-*Mff* stone tool users were mostly adults and subadults, and 88% were males which might be because of the higher weight of stones used. To understand the association between gut-brain axis and stone-tool use behavior, plasma tryptophan (Trp) and serotonin (5-HT) levels were subsequently determined using the HPLC technique and compared between the *Mff* non-stone-tool users and the *Mfa* stone-tool users living in mangrove forests. Because of the very low levels of 5-HT in plasma, the current HPLC method was unable to detect it, and only Trp levels were compared in this study. Categorizing animals into three age-classes (adult, subadult and juvenile), plasma Trp levels were not significantly different within and between populations, except that the *Mfa* adults had higher plasma Trp levels than the *Mff* adults which were associated with the higher prevalence of *stone-tool use*. Thus, this study revealed an association of gut microbiota, dietary adaptations, and cultural behaviors, particularly stone-tool use, in these macaques, contributing to the knowledge of complex interactions between host genetics, diet, and gut microbiota.

Field of Study:	Zoology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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Raza Muhammad

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Celsius
16S rRNA	16S ribosomal RNA
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
AAAD	Aromatic Amino Acid Decarboxylation
ACTH	Adrenocorticotropic Hormone
ANS	Autonomic Nervous system
ASP	American Society of Primatologists
BBB	Blood Brain Barrier
BIC	Bayesian information criterion
BTB	Bang Ta Boon
BW	Body weight
cm^3	Cubic centimetre
CNS	Central Nervous System
COVID-19	Corona virus disease of 2019
CRF	Corticotrophin Releasing Factor
DNA	Deoxyribonucleic acid
ECs	Enterochromaffin Cells
ENS	Enteric Nervous System
ESS	Effective sample size
GBA	Gut-brain axis
GI	Gastrointestinal

HPA	Hypothalamus Pituitary Adrenal
HPLC	Higher Performance Liquid Chromatography
HVSI	Hypervariable segment I
IACUC	Institutional Animal Care and Use Committee
IL	Interleukin
KPE	Koh Ped
LAT	L-Type Amino Acid Transporter
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
Leu	Leucine
LPS	Lipopolysaccharide
MAO-A	Monoamine Oxidase A
MCMC	Markov Chain Monte Carlo
Mfa	*Macaca fascicularis aurea*
Mff	*Macaca fascicularis fascicularis*
MFRC	Mangrove Forest Research Center
MGB	Microbiota-Gut-Brain
Min	Minute
ML	Maximum likelihood
Mm	*Macaca mulatta*
mmol	millimolar
mtDNA	mitochondrial DNA
NaCl	Sodium Chloride
NGS	Next Generation Sequencing
NHP	Non-Human Primate

NPRCT-CU	National Primate Research Center of Thailand-Chulalongkorn University
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PNY	Piak Nam Yai
r	regression
RNA	Ribonucleic acid
SCFAs	Short Chain Fatty Acids
SDS	Sodium dodecyl sulphate
sec	second
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package for Social Sciences
SRY	Sex determining Y-chromosome
Tph	Tryptophan Hydroxylase
Tris-HCl	Tris hydrochloride
tRNA	Transfer RNA
Trp	Tryptophan
Tyr	Tyrosine
USA	United States of America
v/v	Volume/Volume
Val	Valine
w/v	weight/volume
µg/ml	microgram per milliliter
µL	microliter

μmol	micromolar

CHAPTER I

GENERAL INTRODUCTION

Macaca fascicularis (long-tailed macaque or cynomolgus macaque) has the second-largest distribution among all 439 non-human primate (NHP) species (Estrada et al., 2018). *M. fascicularis* is widespread across Southeast Asia, including continental, peninsular, and insular regions in Myanmar, Thailand, Cambodia, Laos, Vietnam, Malaysia, Indonesia, the Philippines, and India (Nicobar Islands) (Fooden, 1995). They inhabit a diverse range of habitats, including lowland forests, disturbed and secondary rainforests, nipa palm and mangrove riverine and coastal forests. Based on the current classification of *M. fascicularis*, there are 10 recognized subspecies according to their distribution range and physical characteristics (Fooden, 1995). Among these 10 subspecies, *M. fascicularis aurea* (*Mfa* or Burmese long-tailed macaque) is one of the four non-human primates that use stone-tool to access encased food such as oysters and nuts (Malaivijitnond et al., 2007). *Mfa* originates in Myanmar and distributes southwardly to southwestern Thailand through the Mergui Archipelago, where they live in close contact with another subspecies of *M. fascicularis*, *M. f. fascicularis* (*Mff* or common long-tailed macaque). It needs to be noted that *Mff* has never been reported to use stone-tool either in captivity or in their natural habitats (Bandini & Tennie, 2018; Fooden, 1995; Malaivijitnond et al., 2007). Considering the genetic relationship between *Mfa* and *Mff*, the *Mfa* is genetically distant from *Mff* based on the mitochondrial DNA (mtDNA), Y-chromosome *SRY* and *TSPY* genes (Bunlungsup et al., 2016; Matsudaira et al., 2018), whole genome sequence (Osada et al., 2021), and autosomal single nucleotide polymorphisms (SNPs) analysis (Phadphon et al., 2022). Thus, the genetic characteristics might be

one of the clues for the emergence and development of the stone-tool use behavior in *Mfa*. Apart from genetics, the environment and cultural transmission (or learning/cognition) can also contribute to the stone-tool use behavior.

Over the past few decades, researchers have made significant efforts to understand the evolutionary processes that led to the unique features of the hominin brain. Hominin evolution is characterized by a significant increase in relative brain size and complexity, with the brain eventually becoming much larger than that of other primates (Schoenemann, 2006). This trend started with *Homo habilis*, who had a brain size of 600 cm^3 and started manufacturing and using Oldowan stone-tools about 2.6 million years ago. The trend continued with *H. erectus*, which had a brain size of 800 cm^3 and used Acheulean stone-tools. The brain expansion reached its maximum size of about 1,500 cm^3 in *H. sapiens neanderthalensis* and *H. sapiens sapiens* when they manufactured and used Mousterian stone-tools. This expansion occurred rapidly, tripling in size within just 2 million years compared to the other non-human primates whose brain sizes mainly remained the same. These findings suggest that over the past 2 million years, the significant expansions in *Homo*'s brain sizes coevolved with the development of the stone-tool technology (Bretas et al., 2019). Looking back to the *Mfa* and *Mff*, this leads to the hypothesis that the presence of stone-tool use behavior only in the *Mfa* should be attributed to the differences in brain development between the *Mfa* and *Mff*, which might be occurred through the Microbiota-Gut-Brain (MGB) axis.

The gut microbiota is a complex ecosystem of microorganisms that reside in the digestive tract of humans and other animals. It consists of trillions of microorganisms, including bacteria, viruses, fungi, and other microbes (Gill et al.,

2006; Whitman et al., 1998). Bacteria comprise more than 99% of microorganisms in the gut, and more than 1,000 species have been reported to be present in the gut (Qin et al., 2010). The gut microbiota plays various vital roles in the host's body, including protection against pathogens by colonizing mucosal surfaces and producing various antimicrobial substances (Baümler & Sperandio, 2016), regulating the immune system (Gensollen et al., 2016), playing a significant role in metabolism and digestion (Nieuwdorp et al., 2014), controlling epithelial cell proliferation and differentiation, maintaining insulin resistance and its secretion, and influencing gut-brain communication, brain development and behavioral functions (Cryan & Dinan, 2012; Heijtz et al., 2011).

The gut-brain communication is bidirectional with multiple pathways of interactions. For the brain, it accounts for the central nervous system (CNS), including the brain and spinal cord, enteric nervous system (ENS), sympathetic and parasympathetic arms of the autonomic nervous system (ANS), and hypothalamic pituitary adrenal (HPA) axis. For the gut, the gastrointestinal (GI) tract serves as a scaffolding for these pathways (Forsythe et al., 2010). The HPA axis is an important physiological mechanism that helps the body respond to stress. It is a crucial part of the limbic system, which plays a key role in emotional processes and memory. When the body experiences stress or inflammation, this system is activated, and the hypothalamus releases a corticotropin-releasing factor (CRF). The CRF stimulates the pituitary gland to produce adrenocorticotropic hormone (ACTH), which then triggers the release of cortisol from the adrenal glands. Consequently, cortisol stimulates immune cells to secrete cytokines and affects the gut permeability and barrier function and the composition of gut microbiota, and, in turn, the cytokines affect the CNS. In

addition, one of the essential components in this bidirectional communication system is tryptophan (Trp), which is metabolized by the gut microbiota to produce various molecules, especially serotonin which can influence behavior and cognition (Agus et al., 2018; Cryan & Dinan, 2012; Grenham et al., 2011; O'Mahony et al., 2015).

Trp is one of the nine essential amino acids that the body cannot synthesize but must obtain from dietary sources (McMenamy, 1965). Trp is a precursor for several metabolites, most noticeably the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT), which is important in both the ENS and the CNS (Le Floc'h et al., 2011; Meneses, 1999). 5-HT plays a key role in brain function, including the regulation of behavior and cognition (Meneses, 1999; O'Mahony et al., 2015). 5-HT is mainly synthesized and secreted by the enterochromaffin cells in the ENS, which is involved in various GI functions (Kim & Camilleri, 2000). Thus, Trp and 5-HT are identified as epigenetics on brain development (Jenkins et al., 2016). Excluding the effects of genetics and environment on stone-tool use behavior in *Mfa*, epigenetics might be one of the factors associated with their brain development.

Although several studies have investigated the gut microbiota of *M. fascicularis*, those studies were mainly focused on the *Mff* subspecies either in captivity or natural habitat (Cui et al., 2019; Koo et al., 2019; Sawaswong et al., 2023; Sawaswong et al., 2020; Sawaswong et al., 2019; Sawaswong et al., 2021), but not the *Mfa* subspecies. This thesis aims to understand if the habitat types (or diets) and/or host genetics affect the composition of gut microbiota and the levels of Trp and 5-HT. Here, the gut microbiota of the two subspecies of *M. fascicularis* (*Mfa* and *Mff)* living in two different habitat types (island and mangrove) were determined and compared.

The plasma levels of Trp and 5-HT of the mangrove populations of *Mfa* and *Mff* were also analyzed and compared.

Objectives

1. To compare the gut microbiota between two wild populations of each of *Mff* and *Mfa* living on island and mangrove.
2. To assess the phylogenetic diversity of gut microbes in wild *Mff* and *Mfa* populations.
3. To assess the plasma levels of Trp and 5-HT in *Mff* and *Mfa* mangrove populations.

CHAPTER II

LITERATURE REVIEW

1. *Macaca fascicularis*

Macaca fascicularis had the widest geographic distribution among any primates, only next to *Homo sapiens* and *M. mulatta*. They are distributed throughout the mainland and insular Southeast Asia, between 20° N and 10° S, including Myanmar, Thailand, Cambodia, Laos, Vietnam, Malaysia, Singapore, Indonesia, Brunei, and the Philippines (Fooden, 1995). They inhabited various habitat types, such as seashores, riverbanks, mangroves, swamp forests, and primary and secondary forests (Fooden, 1995). According to the geographical distribution and morphological characteristics, *M. fascicularis* was classified into 10 subspecies; *Mff, Mfa, M. f. philippinensis, M. f. umbrosa, M. f. fusca, M. f. lasiae, M. f. atriceps, M. f. condorensis, M. f. tua* and *M. f. karimondjawe* (Fooden, 1995). Among these 10 subspecies, *Mff* had the widest distribution throughout mainland Southeast Asia and the islands of Indonesia. The second widespread subspecies is *Mfa*, which was found in Myanmar and southwestern Thailand along the Mergui Archipelago. In the past, they were also reported in Bangladesh, but because of human disturbance, they recently became extinct. The third most geographically distributed subspecies is *M. f. philippinensis*, which was found in the Philippine islands, except for the western region of Mindanao, where *Mff* inhabits. The remaining seven subspecies inhabit only small deep-water fringing islands. Here, this thesis focuses only on *Mff* and *Mfa* subspecies.

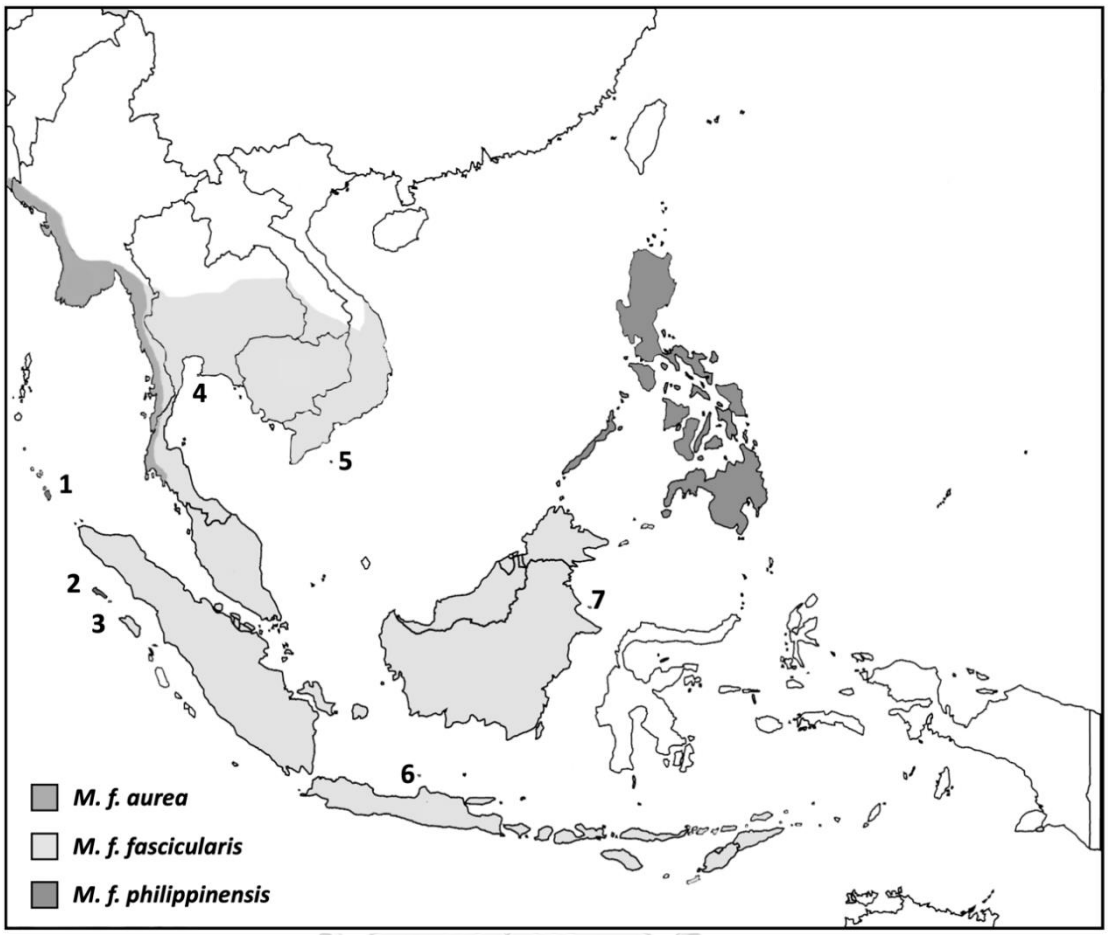

Figure 2. 1. The geographic distribution of 10 *Macaca fascicularis* subspecies. The distribution range of *Mfa, Mff,* and *M. f. philippinensis* subspecies are highlighted in dark grey, light grey, and black color, respectively. Numbers denote the other subspecies; **1**. *M. f. umbrosa,* **2**. *M. f. fusca,* **3**. *M. f. lasiae,* **4**. *M. f. atriceps,* **5.** *M. f. condorensis,* **6**. *M. f. tua* and **7**. *M. f. karimondjawe* (Gumert, 2011).

1.1. Morphological characteristics of *Mfa* and *Mff*

The lateral facial crest pattern was the key morphological characteristic to identify *Mfa* and *Mff* (Fooden, 1995). *Mfa* had a distinct facial crest hair of

infrazygomatic pattern that the hair in the temporal area flew smoothly from the posterior end of the eye to the interior side of the ear, sometimes forming a whorl hair pattern below the zygomatic bone. *Mff* had a transzygomatic crest hair pattern that the crest sweeps upward from the angle of the jaw to the lateral margin of the crown by crossing over the zygomatic bone (Fig. 2.2) (Fooden, 1995). In general, *Mfa* had a darker appearance, particularly on their face and nose, than *Mff* (Bunlungsup et al., 2016; Fooden, 1995). Besides the cheek hair pattern, no head crest was found in *Mfa*, but it might be present or absent in *Mff*.

With the zoogeographic barrier known as the Isthmus of Kra (approximately 10° 30′N), two distinct forms of *Mff* were observed in the northern and southern regions, as noted by Hamada et al. (2008) in terms of their morphological characters, and Tosi et al. (2002) in terms of their genetic makeup (Hamada et al., 2008; Tosi et al., 2022). These two forms were categorized as Indochinese and Sundaic *Mff*. The Indochinese region encompasses mainland Southeast Asian countries such as Myanmar, Vietnam, Laos, Cambodia, and most of Thailand, excluding its southern peninsula. On the other hand, the Sundaic region consists of peninsular Thailand, Malaysia, and the islands of Sumatra, Java, Borneo, Bali, and the Lesser Sunda Islands until Timor Island. Specifically, the Sundaic form of *Mff* exhibited a longer tail and a darker pelage color (Hamada et al., 2008).

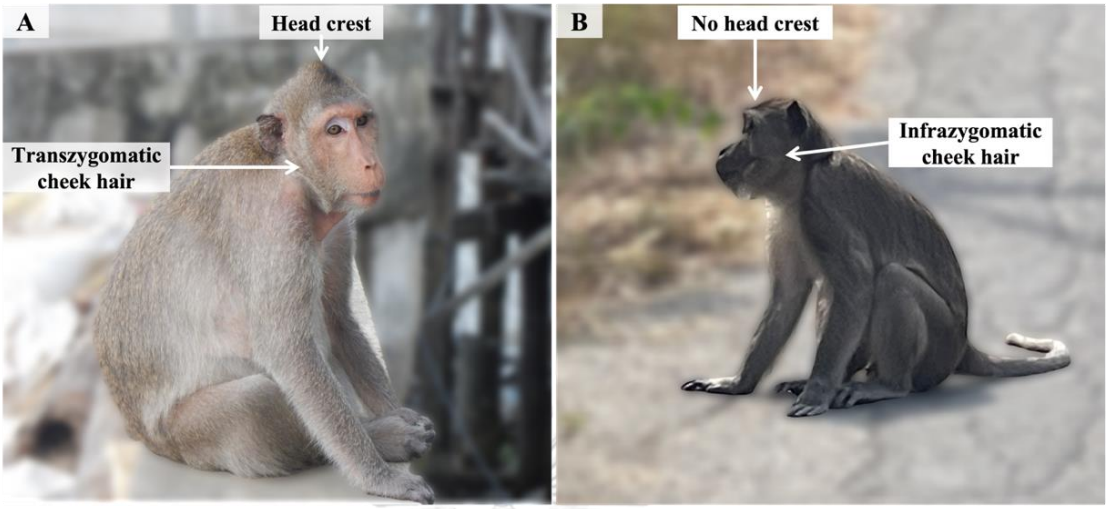

Figure 2. 2. Morphological characteristics of *Mff* and *Mfa*. **A**) *Mff* has a transzygomatic cheek hair pattern with a head crest, **B**) *Mfa* has an infrazygomatic cheek hair pattern without a head crest.

1.2. Genetic characteristics of *Mfa* and *Mff*

In Bunlungsup et al. (2016)'s study analyzing mtDNA and Y chromosome gene sequences, *Mfa* and *Mff* had genetic differences that *Mff* and *M. mulatta* were more closely related to each other than the *Mfa* (Fig. 2.3).. The genetic distinction between *Mfa* and *Mff* was later confirmed by the 868 Restriction Site Associated DNA Sequencing (RADseq)-derived autosomal SNP markers (Phadphon et al., 2022). Further analysis of the whole mtDNA genome sequences indicated that the *Mfa* clustered within the *sinica* species group (including *M. sinica* or Bonnet macaque) but not in the *fascicularis* species group (including *Mff* and *M. mulatta*) (Matsudaira et al., 2018; Fig. 2.4).

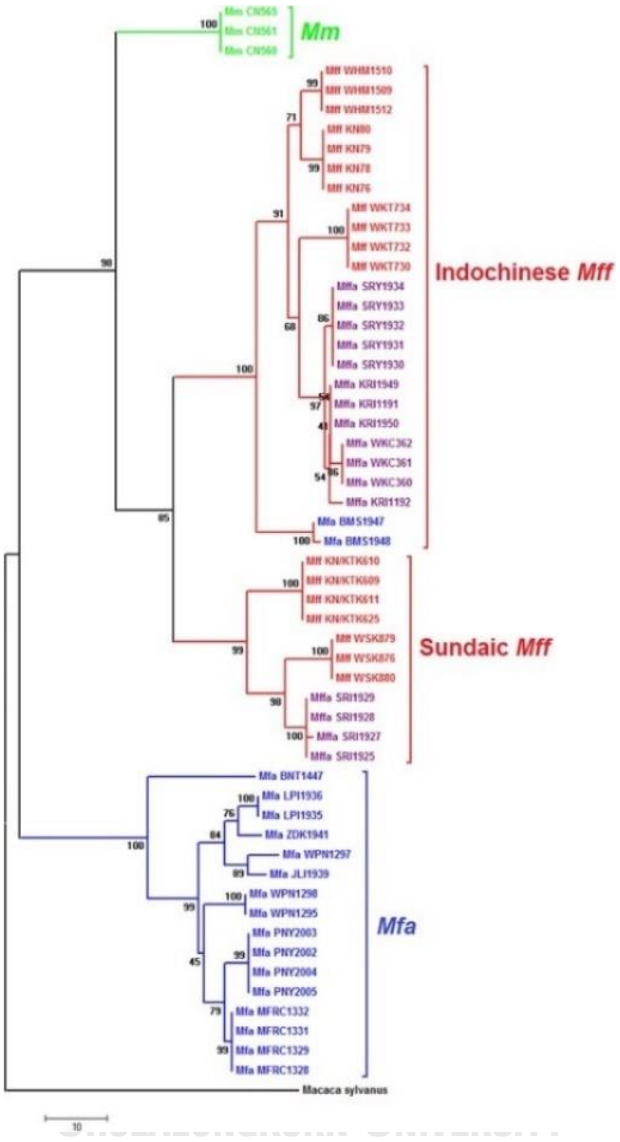

Figure 2. 3. The phylogenetic tree is based on 677 bp of mtDNA. The green, red, blue, and purple letters indicate *M. mulatta*, *Mff*, *Mfa*, and a hybrid between *Mff* and *Mfa*, respectively (retrieved from Bunlungsup et al., 2016).

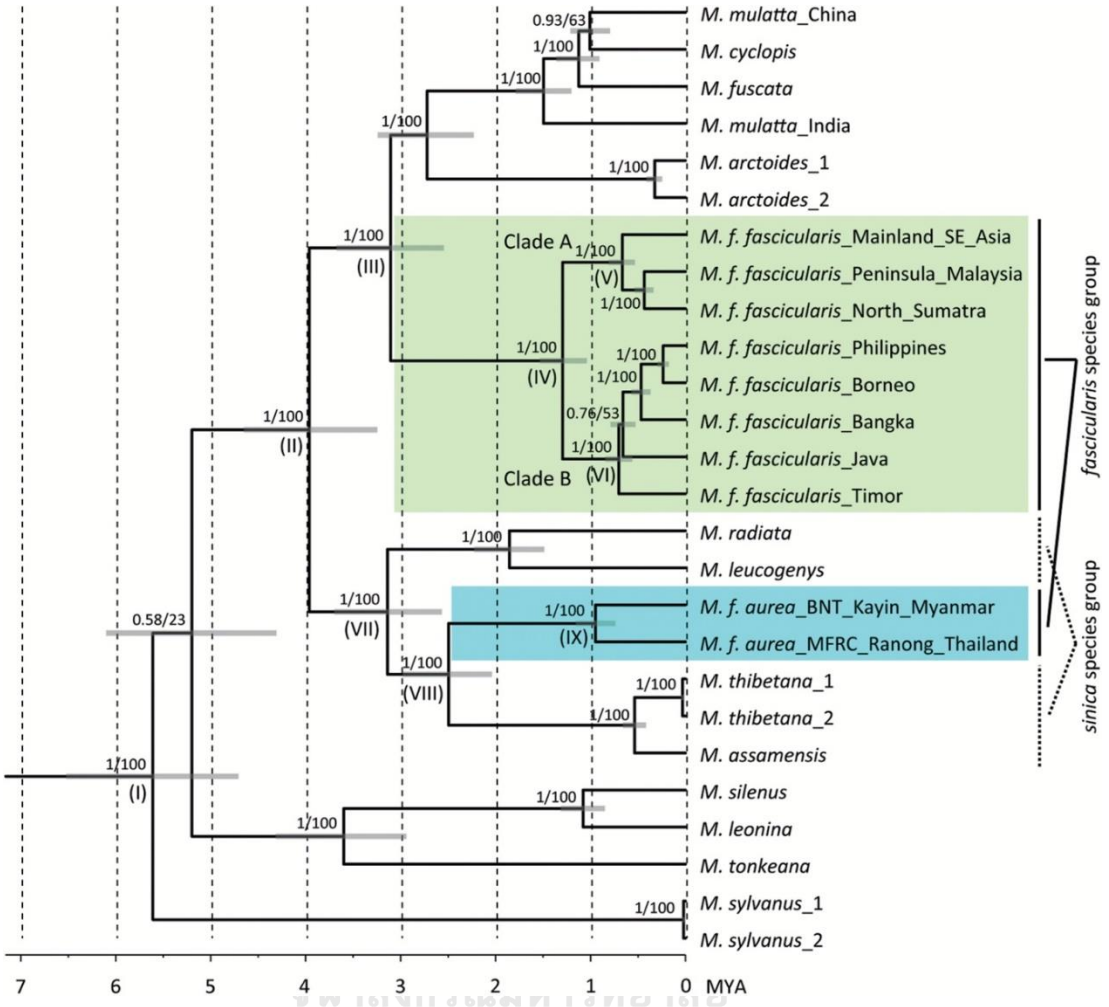

Figure 2. 4. The phylogenetic tree of the whole mitochondrial genome of *Mfa*, *Mff* and other macaque species (retrieved from Matsudaira et al., 2018).

1.3. Hybridization between *Mfa* and *Mff*

Based on the geographic distribution of *Mfa*, they lived in close contact with *Mff* in southwestern Thailand, and a hybrid between the two subspecies was reported (Bunlungsup et al., 2016; Gumert et al., 2019; Phadphon et al., 2022). Hybrids could be distinguished by their physical traits on a combination of different morphological features. The hybrids had a mix of both patterns on each side of the cheek, or asymmetrical patterns of cheek hair presenting on each side of the cheek; a *Mff*'s transzygomatic pattern presenting on one side of the cheek and a *Mfa*'s infrazygomatic pattern on the other side (Fig. 2.5) (Bunlungsup et al., 2016). The genetic studies using mtDNA, Y-chromosome genes, and whole-genome sequences have confirmed the occurrence of hybridization between the two subspecies at 8°10'-12°24'N (Bunlungsup et al., 2016; Phadphon et al., 2022; Figure 2.6). The hybridization predominantly involved the introgression of genetic material from *Mfa* to *Mff*, primarily male-mediated introgression (Bunlungsup et al., 2016; Matsudaira et al., 2018; Osada et al., 2021; Phadphon et al., 2022). The stone-tool use behavior was also reported in the hybrid populations, i.e., at Koram Island and Nom Sao Island, Khao Sam Roi Yot National Park, Prachuap Khiri Khan province, Thailand (Lydia V Luncz et al., 2017).

Figure 2. 5. Mixed cheek hair pattern of *Mfa* x *Mff* hybrid (Klong Mudong population; 7°50'N, 98°22'E).

Photograph credit; Poompat Phadphon.

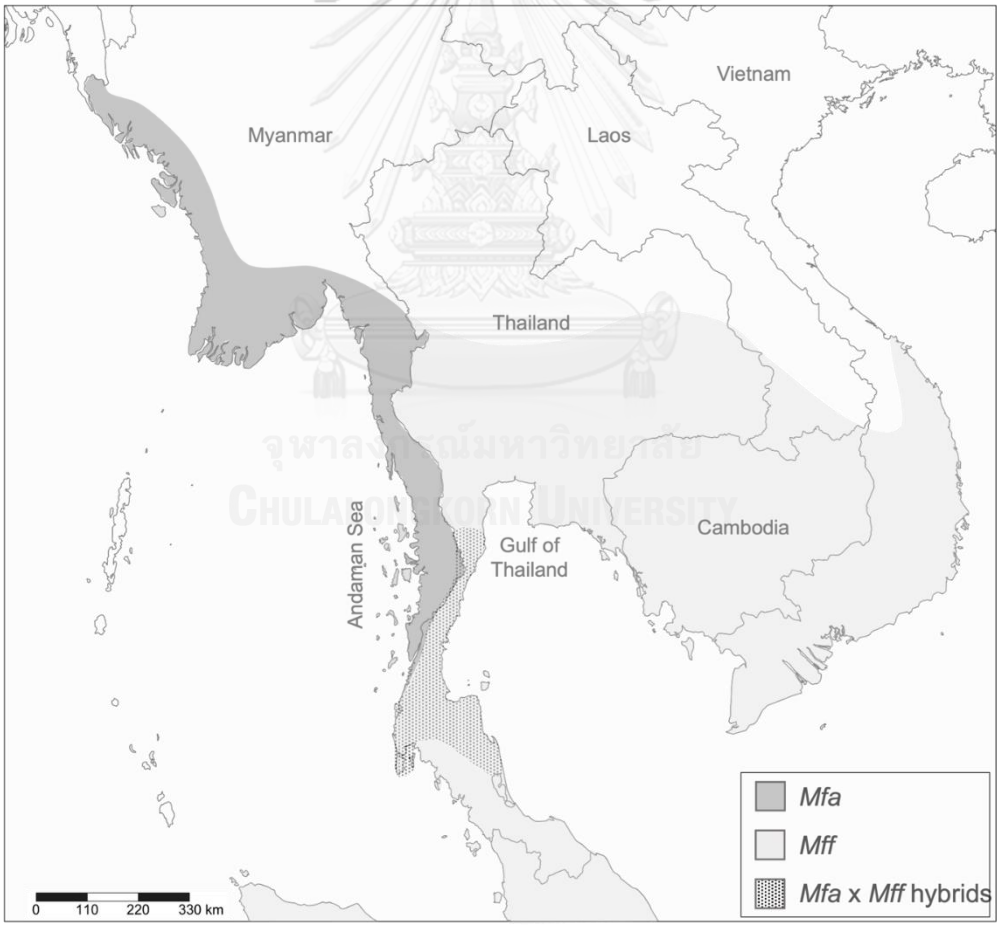

Figure 2. 6. The distribution range of *Mfa* (dark grey), *Mff* (light grey), and their hybrids (dot) (Bunlungsup et al., 2016; Fooden, 1995).

1.4. Stone-tool use behaviors

Among 10 subspecies of *M. fascicularis*, *Mfa* (Malaivijitnond et al., 2007) has acquired attention since 2007 that they used percussive stone-tools to open encased foods such as hooded rock oysters (*Saccostrea cucullate*), sea almonds (*Terminalia catappa*), nuts, and shellfish in their natural habitats (Gumert et al., 2011; Gumert et al., 2009; Gumert & Malaivijitnond, 2012, 2013; Malaivijitnond et al., 2007). Considering the existing data, the prevalence of stone-tool use behavior in *Mfa* was significantly high. By comprehensive surveys of macaques conducted across Thailand; however, no stone-tool use behavior in *Mff* was reported (Malaivijitnond & Hamada, 2008; Malaivijitnond et al., 2005).

Gumert et al. (2019) discussed how multiple factors, such as ecological, cultural, and inherited factors, can impact the emergence and progression of stone-tool use behavior. For ecological factors, *Mfa* populations exhibited a higher tendency to inhabit coastal and estuary habitats than the overall *Mff* populations. These habitats offered abundant encased food resources and pebble stones, making *Mfa* favorable for the selection of stone-tool use behavior through natural selection. Particularly, a population faced ecological challenges while foraging for their nutritional needs in this habitat. For cultural and social factors, the presence of role models within social groups and the influence of their activities on shaping the environment play a role in this process (Gumert et al., 2019).

2. The gut microbiota

"Microbiota" is the collection of microorganisms which includes bacteria, viruses, fungi, archaea, and protists, while "microbiome" refers to the collective genome contents of microbiota. However, microbiota and microbiome are frequently used interchangeably. In humans, the microbiota can be found within the tissues and biofluids, including skin, mammary glands, seminal fluid, uterus, ovarian follicles, lung, saliva, oral mucosa, conjunctiva, biliary tract, and GI tract; however, the highest microbial density of the microbiota was in the colon which is a part of the GI tract (Guarner & Malagelada, 2003; Whitman et al., 1998). Microbiota in the human body is estimated to be up to 100 trillion cells (10^{14}), ten-fold higher than the number of human cells and encoded 100-fold more unique genes than the human's genome (Gill et al., 2006; Whitman et al., 1998). Noted, a newly revised estimate indicated that the human and bacterial cell ratio was closer to 1:1 (Sender et al., 2016). Microbiota contained > 99% of bacterial genes, approximately 1,000 to 1,150 bacterial species (Qin et al., 2010), and a large proportion of the fecal mass harboring bacteria (around 60% of fecal mass) (Stephen & Cummings, 1980). Therefore, the human gut and other mammals are a significant microbial ecosystem in the biosphere.

2.1. Composition of gut microbiota

Firmicutes and Bacteroidetes were the two most abundant bacterial phyla comprising approximately 90% of the gut microbiota, while the other bacterial phyla, such as Actinobacteria, Proteobacteria and Verrucomicrobia, were less abundant, as shown in Table 2.1 (Bidell et al., 2022; Falony et al., 2016; Huttenhower et al., 2012). However, the relative proportion of each taxon varies individually. More than 500

different genera and 1,000 species of bacteria were identified across human populations providing high diversity of lower taxonomic levels (Falony et al., 2016; Huttenhower et al., 2012). At the genus level, a “core” human gut microbiota, defined as genera shared by ≥95% of individuals, included only 14 genera (Falony et al., 2016). However, a healthy adult commonly hosted > 100 bacterial species in their GI tract, with genus and species compositions demonstrating substantial interindividual variation (Huttenhower et al., 2012; Qin et al., 2010). Within this diversity of gut microbiota, it is a tremendous genetic potential of substantial functional redundancy that includes myriad functions not found in the human genome (Huttenhower et al., 2012; Qin et al., 2010).

Table 2. 1. Overview of the four most common bacterial phyla within the gut microbiome in healthy human individuals.

Phylum	Description	Genus examples
Bacteroidetes	Gram-negative; typically obligately anaerobic; often abundant	Bacteroides, Prevotella
Firmicutes	Gram-positive; typically obligately anaerobic; often abundant; highly diverse phyla	Clostridium, Enterococcus, Eubacterium, Faecalibacterium, Lactobacillus, Roseburia, Ruminococcus, Streptococcus
Actinobacteria	Gram-positive; typically obligately anaerobic/requires low oxygen levels	Bifidobacterium, Corynebacterium, Eggerthella
Proteobacteria	Gram-negative; mostly facultatively anaerobic; includes many pathogenic species	Enterobacter, Escherichia, Klebsiella, Serratia

The genera Lactobacillus and Bifidobacterium were the two most common examples of beneficial microbes (Table 2.2). These genera included strains that were

accounted for as “probiotics”, live microorganisms that provided a benefit to the host's health when administered in appropriate ratios (Hill et al., 2014; Karl et al., 2018). They were also the only two genera that were historically identified as beneficial microorganisms in the prebiotic concept, which emphasizes selective activation of Lactobacillus and Bifidobacterium development as a beneficial human health effect (Gibson et al., 2017; Roberfroid et al., 2010). The strains from these genera enhanced immunological function, assisted digestion, inhibited pathogen colonization, and modified GI physiology positively (Hill et al., 2014). Notably, the genera Faecalibacterium, Eubacterium, and Roseburia were also recognized as beneficial microbes (Gibson et al., 2017; Roberfroid et al., 2010). They synthesized the short-chain fatty acid (SCFA) butyrate, which had several extraintestinal and intraintestinal health benefits, such as improving gut barrier integrity and reducing inflammation and oxidative stress (Canani et al., 2011).

The harmful microbes were on the other side of the spectrum. Although many commensal bacteria would be detrimental if they entered systemic circulation, a taxonomic dominance may be undesirable. Enterobacteriaceae, a family that included the gut commensals Shigella, Escherichia, Klebsiella and Proteus, was associated with the development of inflammation and associated diseases (Huttenhower et al., 2014). The underlying mechanism included a formation of lipopolysaccharide (LPS), a common endotoxin attached to the cell surface membrane of gram-negative bacteria, stimulated the immune system and triggered a severe pro-inflammatory response (Hurley, 1995).

Table 2. 2. The health-promoting and health-compromising features and functions of gut microbiota.

Characteristics	Effect
Health-promoting	
High species/genetic diversity	Associated with better health and resilience to perturbation
Bifidobacterium (phyla: Actinobacteria), Lactobacillus (phyla: Firmicutes)	Genera commonly used in probiotics; linked to multiple favorable health effects, including increased resistance to infection and diarrheal disease, immune-enhancement, anti-carcinogenic, vitamin production, and secretion of anti-microbial compounds
Roseburia, Eubacterium, Clostridium clusters XIVa and IV (phyla: Firmicutes) Faecalibacterium prausnitzii (phvla: Firmicutes) Increased butyrate production	Butyrate producers Anti-inflammatory, butyrate producer Major energy source of colonocytes, anti-inflammatory, regulates cell growth and differentiation, anti-carcinogenic, improved gut barrier function, reduced colonic pH
Carbohydrate fermentation/increased short-chain fatty acid (butyrate, acetate, propionate) production	Reduced colonic pH, pathogen inhibition, the anti-inflammatory, anti-carcinogenic energy source for peripheral tissues, enhanced mineral absorption
Health-compromising	
Low diversity/high pathogen load	Compromised gut barrier integrity, local and systemic inflammation
Proteobacteria (includes family Enterobacteriaceae) Protein fermentation	Phyla, which produces pro-inflammatory lipopolysaccharide Production of potentially carcinogenic/toxic compounds (N-nitroso compounds, amines, p-cresol, NHs, phenols, amines, thiols)
Sulfate/sulfite-reducing bacteria, e.g., Bilophila wadsworthia, Desulfovibrio (phyla Proteobacteria) Mucin degradation > synthesis	Production of toxic H_2S Compromises gut barrier integrity, facilitates bacterial translocation to the epithelium, provides sulfates for H_2S

2.2. The roles of gut microbiota

The microbiota served a variety of physiological ways in the host, including enhancing gut integrity or shaping intestinal mucosa (Natividad & Verdu, 2013), providing energy (Den Besten et al., 2013), helping to prevent pathogens (Baümler & Sperandio, 2016), and modulating host immune system (Gensollen et al., 2016). The fermentation of non-digestible dietary residues and endogenous mucus produced by the epithelia was also the key metabolic role of gut microbiota (Brubaker, 2018; Nieuwdorp et al., 2014). The microbial provided several enzymes and metabolic pathways that were distinctive from the host's innate constitutive resources (Le Chatelier et al., 2013), resulting in the recovery of metabolic energy and absorbable substrates for the host. The following are examples of the role of gut microbiota in the hosts.

2.2.1. Immune modulation

Gut microbiota appeared to help both adaptive and innate immunity through various complex and poorly understood pathways. In general, the host immune system and the microbiota interacted through several mechanisms to influence both anti- and pro-inflammatory responses in the gut (Kho & Lal, 2018). In a healthy condition, commensal microbiota appeared to aid the host immune system in identifying friend or foe microorganisms, helping to either moderate or trigger an inflammatory response, respectively. The microbiota was also known to interact with gut T lymphocytes to maintain a balance between immune-suppressing regulatory and immune-stimulating T helper cells and the immunoglobulin A secretion in the gut (Kamada & Núñez, 2013). *Bifidobacterium infantis*,

Bacteroidetes (i.e., *B. fragilis*), and Firmicutes (i.e., *Clostridia species*) were all associated with the recruitment of regulatory T-cells. Also, the *Faecalibacterium prausnitzii* appeared to enhance levels of anti-inflammatory cytokines (e.g., IL-10) and reduced levels of pro-inflammatory cytokines (i.e., IL-12) (Kho & Lal, 2018). Therefore, the gut microbiota seems to assist the preservation of healthy gut epithelium by reducing the inflammation and enhancing the induction of a suitable inflammatory immune response (Wu & Wu, 2012).

2.2.2. Short-chain fatty acid production

The gut microbiota produced SCFAs from the host diets containing unabsorbed starches and dietary fiber (Dalile et al., 2019; Tan et al., 2014). Diets high in fiber (e.g., plant-based) or fermented foods were considered "gut healthy" as they produced SCFA. SCFA contains various immune-modulating characteristics, including the capacity to interact with neutrophils to either stimulate or reduce local activity and the potential to neutralize the proinflammatory effect of specific cytokines on the gut epithelium. Therefore, SCFA plays a key role in maintaining the health of the gut barrier by providing epithelial cells with energy and reducing local inflammation (Morrison & Preston, 2016). Butyrate, propionate, and acetate were the three most predominant SCFAs. The production of butyrate was particularly important because it was a preferred energy source for colonic epithelial cells (Frankel et al., 1994), and could even induce apoptosis in colon cancer cells (Ruemmele et al., 1999). The reduction in butyrate might cause intestinal inflammation (Harig et al., 1989). Butyrate synthesized by gut microbiota was known to contribute 5 – 10% of the host's caloric requirements via providing energy to colon

cells (Lawley & Walker, 2013). Propionate also serves as an energy source for epithelial cells. It was transported to the liver and transformed to glucose via the gluconeogenesis pathway, which substantially enhanced energy homeostasis by reducing hepatic glucose synthesis thereby decreasing adiposity (Brown et al., 2003). Acetate was one of the most abundant SCFAs, which was a crucial co-factor/metabolite for other bacteria's growth; for example, in the absence of acetate, *Faecalibacterium prausnitzii* could not grow in pure culture (Duncan et al., 2004).

2.2.3. Vitamin synthesis

The gut microbiota was essential for the synthesis of various vitamins, such as vitamins B and K. The endogenous B vitamins, i.e., cyanocobalamin, were mainly synthesized by the gut microbiota (Actinobacteria, Firmicutes and Proteobacteria strains) and were necessary for a variety of healthy metabolic activities such as replication and repairing of DNA (Das et al., 2019; Hill, 1997). *Bacteroides fragilis, Eubacterium lentum, Enterobacter agglomerans, Serratia marcescens*, and *Enterococcus faecium* were among the commensal bacteria that produce vitamin K, and alterations in the abundances of these microorganisms can thus affect the availability of certain endogenous vitamins (Conly & Stein, 1992; Pham et al., 2021).

2.2.4 Colonization resistance

“Colonization resistance” typically refers to techniques used by the gut microbiota to reduce the risk of localization of potentially harmful microorganisms. It is one of the beneficial characteristics conferred by a vast consortium of gut microorganisms in homeostatic abundances. It also preserved healthy homeostatic

bacterial abundances within the microbiome community (Lawley & Walker, 2013). Several bacteria can also utilize their antimicrobial means to control proliferation and/or to target invading cells. Some commensals produced diffusible proteins known as bacteriocins, which were highly deleterious to surrounding species by triggering pore formation, genomic degradation, and/or disruption in cell wall synthesis (Yiu et al., 2017). Bacteroidetes and Proteobacteria have antibacterial secretory systems that can damage any surrounding cells with different inherited characteristics (Britton & Young, 2012). Although the mechanism of colonization resistance differed, they all acted to reduce the risk of systemic and local infection to the host while aiding in the conservation of gut microbiome homeostasis.

2.3. Dysbiosis of gut microbiota

The microbiota and its host had a complex symbiotic relationship, and the disruption in this relationship may have detrimental effects on both (Thursby & Juge, 2017). Dysbiosis is an alteration of gut microbiota composition and/or function that deviates from a stable or normal condition (Buford, 2017; McBurney et al., 2019). These alterations in microbial composition were believed to contribute to the development of various pathological conditions, such as inflammatory bowel disease (Ferreira et al., 2014), irritable bowel syndrome (Kennedy et al., 2014), obesity (DiBaise et al., 2008), diabetes mellitus (Gurung et al., 2020), cardiovascular diseases (Tang & Hazen, 2014), and neurological disorders (Pellegrini et al., 2018).

3. Factors affecting gut microbiota

3.1. Diet

The diversity and relative abundance of gut microbiota were strongly influenced by the dietary components consumed by the hosts (Fig. 2.7), as revealed by the substantial variations in microbial communities between plant-rich and (animal) protein-rich diets (David et al., 2014). This occurred because nutrient-induced selective pressures on microbiota preferred bacterial species that were rich in genes essential for specific metabolic activities. For instance, a plant-based diet enhanced luminal fiber as well as complex carbohydrates; thereby, species which were enriched in carbohydrate-active enzymes were selected (Desai et al., 2016). While the animal-based diet was enriched in fats and proteins, but having low fibers enhanced luminal bile concentration, which favored bile acid-resistant bacteria harboring bile acid metabolism genes (David et al., 2014; Forouhi et al., 2018). In humans, diet played an important role in shaping the gut microbiota's structure, shape, and diversity after the infant state as it adapted to altered nutrient availability. During the early stages of infancy, the gut microbiota was highly enriched genes involved in the metabolism of oligosaccharides present in breast milk. Later, when solid meals were introduced, the metagenome became enriched with genes involved in the metabolism of vitamins and polysaccharides (Bäckhed et al., 2015).

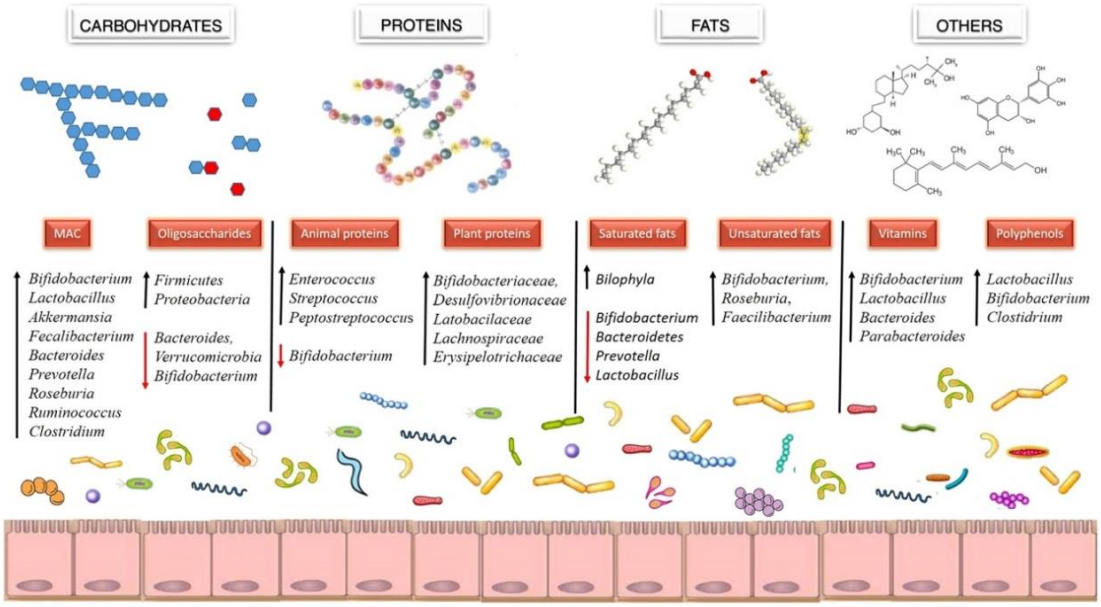

Figure 2. 7. Overview of the main effects of dietary components on the composition of the gut microbiota. The arrows indicate the increase (black) or decrease (red) in bacterial abundance. MAC: microbiota-accessible carbohydrates. Source: (Ramos & Martín, 2021).

3.2. Environment

The environment was perhaps one of the most significant impacts in shaping gut microbiota composition and diversity. Various environmental factors, including habitat type, climate/season, geography, and access to food sources, differ across regions and can impact the types and abundance of microbial species present in the gut. For instance, individuals living in rural areas or traditional societies may have distinct microbiota compositions compared to those in urbanized environments due to differences in diet, lifestyle, and exposure to environmental microbes (De Filippo et al., 2010; Lin et al., 2013). Another study reported that human populations residing in

three different geographic origins (USA, Malawi, and Venezuela) identified significant variations in the composition and diversity of gut microbiota (Yatsunenko et al., 2012). Similar to humans, the diversity of gut microbial composition in free-ranging Tibetan macaques (*M. thibetana*) was significantly influenced by geographical environments, and variations in seasons (winter and spring) that offered different food resources (Sun et al., 2016). The black howler monkey (*Alouatta pigra*) living in Mexico's Palenque National Park modified its dietary preferences based on the cyclic transitions between the rainy and dry seasons. This adaptation involved adjustments in the utilization of plant species and different plant parts. By altering the gut microbial community function, the howler monkeys could sustain relatively stable activity levels despite the seasonal variations in their diets. This observed shift in the gut microbiota compensated for the changes in the monkeys' dietary patterns throughout the seasons (Amato et al., 2015). Another study was conducted on Chinese rhesus macaques residing in six geographical environments at different altitude levels. During the adaptation of rhesus macaques to diverse geographical environments, the abundance of the shared core intestinal microbial flora underwent varying degrees of alteration, leading to the emergence of novel and distinctive microbial communities. This process played a significant role in reshaping the gut microbiota of rhesus macaques. Notably, the observed changes were more pronounced among animals inhabiting high-altitude environments (Zhao et al., 2018).

It is important to note that the influence of the environment on gut microbiota is complex and can vary among individuals due to genetic factors and other individual-specific characteristics. Nonetheless, understanding the interplay

between the environment and the gut microbiota is crucial for promoting gut health and preventing the development of various diseases.

3.3. Host genetics

Host genetics played a significant role in shaping the composition of gut microbiota in humans as well as in NHPs. The genetic makeup of an individual can influence various aspects of the gut microbiome, including the types of microorganisms, their relative abundance, and their metabolic functions. The composition of the microbial community was influenced by the evolutionary relationship among hosts, primarily through immune-related genes that played a role in shaping interactions between hosts and their microbiota. Additionally, the transmission of microbiota from parents to offspring, known as vertical transmission, also contributes to the influence of host phylogeny on the microbial community. A study conducted on a group of 416 twins in the UK demonstrated a strong influence of host genetics on gut microbiota composition (Goodrich et al., 2016). Another study focusing on captive colobine monkeys (*Rhinopithecus brelichi, Rhinopithecus roxellana, Rhinopithecus bieti, Pygathrix nemaeus, Nasalis larvatus, Trachypithecus francoisi, Trachypithecus auratus, Trachypithecus vetulus,* and *Colobus guereza*) highlighted the considerable impact of both diet and phylogeny on the gut microbiota (Hale et al., 2018). Investigating the lineage of primates found that both host physiology and phylogeny significantly affected the gut microbiomes (Amato et al., 2019). The interplay between host genetics, diet, and environmental factors contributed to the complex relationship between the host and its gut microbiome.

4. Gut-brain axis and the bidirectional communication

The bidirectional communication between the gut and the brain is now widely recognized (Cryan & Dinan, 2012). Multiple interconnected pathways were involved, including the neuroendocrine, immune, autonomic, and ENS (Aziz & Thompson, 1998; Banks, 2008; Mayer, 2011). These systems work together to enable the exchange of information between different parts of the body. The primary site of these interactions was the GI tract, which contained approximately 500 million nerve endings and a robust presence of immune cells (Furness et al., 1999). Within the GI tract, the ENS played a key role, with intrinsic primary afferent neurons transmitting subtle changes in the GI tract to the brain via the vagus nerve. Immune cells release cytokines, essential for the body's response to inflammation and infection. Additionally, neuroendocrine hormones, for example cortisol, affect the permeability and barrier function of the gut and communicate with immune cells to regulate cytokine secretion (Fig. 2.8) (Cryan & Dinan, 2012). This intricate signaling process occurred throughout the body, establishing connections between the GI tract and the CNS. Referred to as the "gut-brain axis", this dynamic signaling pathway involves various tissues and organs such as the brain, endocrine glands, gut, immune cells, and GI microbiota. Researchers have extensively studied the components of this axis due to their involvement in digestive function and the regulation of satiety (Konturek et al., 2004; Tachf et al., 1980). Dysfunction in the gut-brain axis can have far-reaching pathological consequences and is associated with conditions including inflammation, chronic abdominal pain, eating disorders, nausea, and stress (Drossman, 1998; Mayer, 2011).

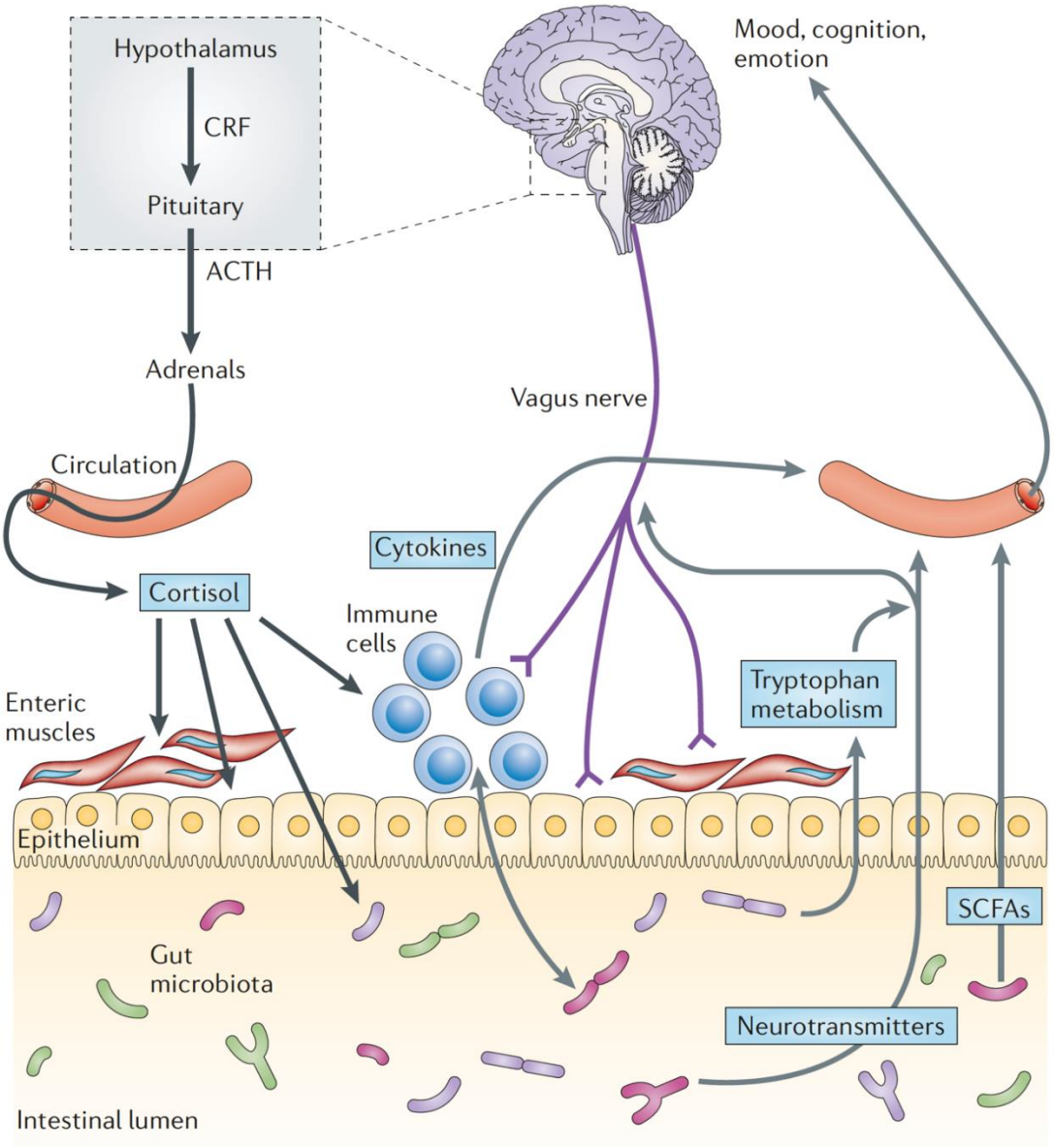

Figure 2. 8. The bidirectional communication pathways between the gut microbiota and the brain.

Abbreviations: ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; SCFA, short-chain fatty acid (Source: Cryan & Dinan, 2012).

4.1. Key communication pathways in gut-brain axis

Research in neuroscience, gastroenterology, and microbiology has yielded evidence supporting the influence of gut microorganisms on a range of metabolic, GI, and neurological diseases (Sherwin et al., 2016). The intricate communication network between the gut microbiota and the brain, facilitated through the gut-brain axis, indicated that these microorganisms could impact brain chemistry and behavior (Cryan & Dinan, 2012; Grenham et al., 2011). The key pathways implicated in this communication and their effects on neurobiology encompassed the vagus nerve, interactions with the cell wall, and Trp metabolism. Gaining a comprehensive understanding of these pathways can provide valuable insights into the effects of the microbiota on maintaining balance within the body and their involvement in complex CNS disorders.

4.1.1 Vagus nerve

The vagus nerve, also known as cranial nerve X, serves both efferent and afferent functions. It plays a crucial role as the primary nerve of the parasympathetic division of the ANS, regulating various organ functions such as bronchial constriction, heart rate, and gut motility (Moriss, 2013). It also had significant anti-inflammatory capabilities, providing protection against microbial-induced sepsis through the involvement of the nicotinic acetylcholine receptor α7 subunit (Wang et al., 2003). Approximately 80% of the nerve fibers within the vagus nerve are sensory, transmitting information about the state of the body's organs to the CNS (Thayer & Sternberg, 2009). Many of the effects observed in the influence of the gut microbiota or potential probiotics on brain function have been found to rely on vagal activation

(Bravo et al., 2011; Goehler et al., 2008). However, it is essential to note that there were also vagus-independent mechanisms involved in microbiota-brain interactions, as vagotomy (surgical removal or disconnection of the vagus nerve) showed no impact on the effects of antimicrobial treatments on the brain or behavior (Bercik et al., 2011). However, the precise mechanisms by which the gut microbiota activates vagal afferents are currently poorly understood.

4.1.2 Cell wall components and immune response

The bacterial cell wall consisted of peptidoglycan, activating innate and adaptive components of the host's mucosal immune system. These responses were initiated by recognizing the inflammatory microbial components known as pathogen-associated molecular patterns (PAMPs). PAMPs are bound to pattern-recognition receptors on immune cells, leading to the production of inflammatory cytokines. These cytokines can indirectly affect the brain through peripheral vagal pathways or directly by affecting permeable areas of the blood-brain barrier (BBB; Sherwin et al., 2016). For instance, Interleukin (IL) 6 and chemokine ligand 2 pro-inflammatory cytokines can influence the brain through two pathways. The first pathway, known as the humoral pathway, involved PAMPs acting on toll-like receptors in specific brain regions. The second pathway involved afferent nerves transmitting signals to the brain. Gram-negative bacteria-associated PAMPs included peptidoglycan monomers, lipopolysaccharide, porins, and mannose-rich sugar chains. Gram-positive bacteria associated with PAMPs included peptidoglycan monomers and lipoteichoic acids. These cell wall components may stimulate the production of additional molecules involved in neural signaling from cells (Forsythe & Kunze, 2013). While further

research is needed to confirm these findings, the strong immunomodulatory effects of the gut microbiota on both mucosal and systemic immune systems suggested potential mechanisms through which the gut microbiota can influence brain function and behavior.

4.1.3 Tryptophan metabolism

As mentioned above, the gut and the brain can communicate together via the microbiota, whereby L-tryptophan (L-Trp) was one of the chemicals used for this communication. L-Trp was an essential amino acid for each living cell for protein synthesis (Le Floc'h et al., 2011; McMenamy, 1965). L-Trp was the only amino acid bound with plasma albumin and circulated in the peripheral circulation in an equilibrium between albumin-bound and unbound forms. Approximately 90% of total plasma L-Trp was an albumin-bound form, producing a complex that cannot pass the BBB; the remaining unbound form circulates freely and can cross the BBB into the brain (McMenamy, 1965; Pardridge, 1979). The L-type amino acid transporter (LAT1) at the luminal and abluminal surface of endothelial cells transported free L-Trp across the BBB. Trp, phenylalanine (Phe), and tyrosine (Tyr) competed for transport by LAT1 with the branched chained amino acids isoleucine (Ile), leucine (Leu), and valine (Val) (Madras et al., 1974). Apart from its role in protein synthesis, L-Trp produced various biologically active metabolites, including 5-HT (Berger et al., 2009; Rapport et al., 1948). 5-HT was an important neurotransmitter in both ENS and CNS (Kim & Camilleri, 2000; O'Mahony et al., 2015). 5-HT had two functions: (i) a growth factor during the early developmental phase, which regulated the development of its own and related neuronal systems, and (ii) a neurotransmitter in the mature

brain which regulated attention, cognition, and emotion (Meneses, 1999). The vast majority of 5-HT was found within the gut, which was synthesized from Trp in the GI's enterochromaffin cells.

The synthetic biochemical pathway for 5-HT initiated with the conversion of Trp to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (Tph) (Fig. 2.9). Tph had two isoforms; Tph1 that was synthesized only to peripheral 5-HT, and Tph2 that was synthesized to a central 5-HT. The conversion of Trp to 5-HTP was a rate-limiting step for the 5-HT synthesis. Subsequently, the 5-HTP was decarboxylated through the aromatic amino acid decarboxylation, leading to 5-HT synthesis (Agus et al., 2018; Kim & Camilleri, 2000). Once the 5-HT was synthesized, it activated the signaling pathways via its 15 receptors classified into 7 families (5-HT1 – 5-HT7) (Meneses, 1999). Finally, the enzyme monoamine oxidase A (MAO-A) catabolized 5-HT to produce 5-hydroxyindole acetic acid (5-HIAA). 5-HIAA was the 5-HT metabolite having no biological activity and excreted out of the body in the urine (Singh et al., 1999).

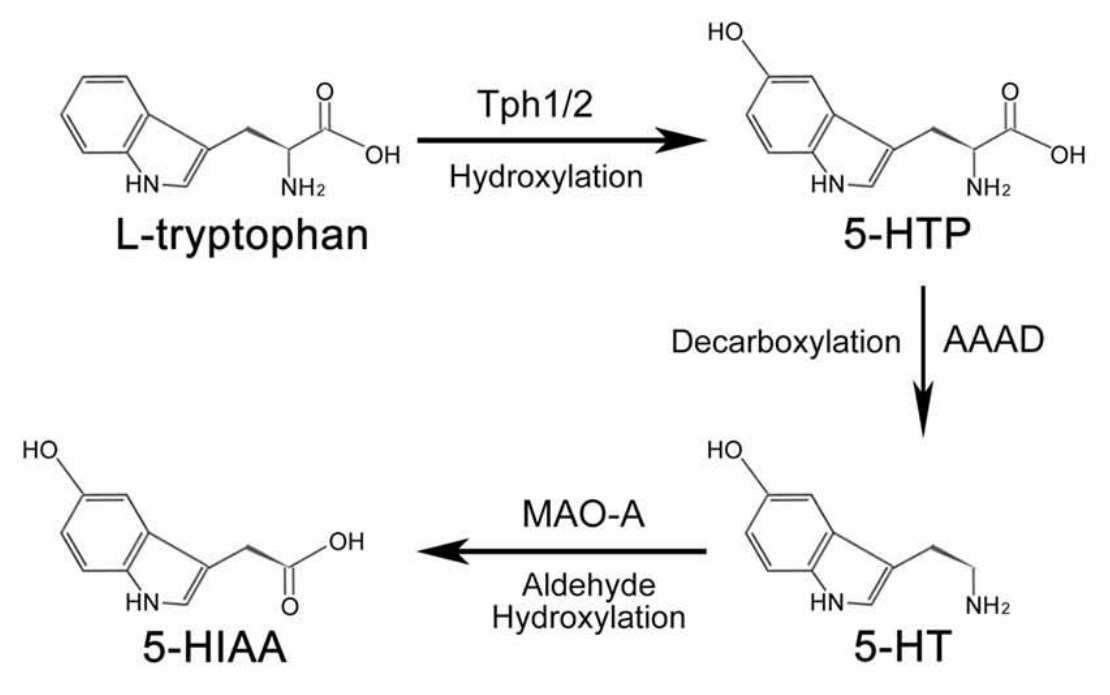

Figure 2. 9. The enzymatic process of the Trp metabolism and the synthesis of 5-HT**.** The Trp is hydrolyzed by the Tph1/2 enzyme producing 5-HTP, which is then decarboxylated by the AAAD to synthesize 5-HT. 5-HT is catabolized by the MAO-A to produce 5-HIAA. Abbreviations: Tph, tryptophan hydroxylase; 5-HTP, 5-hydroxytryptamine; AAAD, aromatic amino acid decarboxylase; 5-HT, serotonin; MAO-O, monoamine oxidase A; 5-HIIA, 5-hydroxyindole acetic acid.

5. Molecular analysis of the gut microbiome

Microbiome research experienced a shift. A decade ago, the knowledge about the microorganisms that resided in various parts of the human body was slightly comprehended. For example, how they formed communities of various complexity and interacted with other microbiomes. In the past, bacterial identification depended only on culture-based methods, which often failed to detect certain bacteria species

that cannot grow in common conditions. These culture-based methods were also challenging when a community of organisms with various individual growth characteristics was studied. The advent of recent advancements in sequencing, data collection, and analysis technologies made these concerns conceivable. These typically started with the amplification and sequencing of specific microbial DNA regions, followed by microbial identification and variation by comparing with the referenced microbial genomic sequences in the databases.

Among sequence-based bacterial analyses, amplicon sequencing of the 16S ribosomal RNA (rRNA) gene has proven to be a reliable and efficient option for the taxonomic classification of microbes (Clarridge, 2004). There are many studies which demonstrated the applications of 16S rRNA gene sequencing in understanding the gut microbiome composition and its relationship with various factors in NHPs (for example, western lowland gorillas (Pafčo et al., 2019), rhesus macaques (Zhao et al., 2018), Tibetan macaques (Sun et al., 2016), black howler monkeys (Amato et al., 2015), and long-tailed macaques (Cui et al., 2019; Koo et al., 2019; Sawaswong et al., 2019, 2020, 2021, 2023)). The 16S rRNA gene was approximately 1,500 bp long, which was highly conserved in prokaryotes and contained hypervariable regions ranging from V1 to V9 flanked by regions of more conserved sequences (Fig. 2.10). These hypervariable regions were frequently used in phylogenetic classifications at genus or species level in diverse microbial populations.

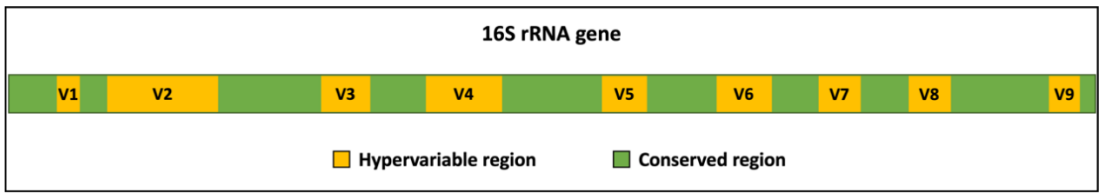

Figure 2. 10. The 16S rRNA gene. Hypervariable (V1–V9) and conserved regions are represented by orange and green colors, respectively.

The very first available 16S rRNA-DNA sequencing technique, which enabled the identification of microbes, was Sanger sequencing (Sanger et al., 1977). With the advent of next-generation sequencing (NGS) technology, parallel high-throughput sequencing of complex microbial communities was massively enabled. The NGS technique is highly sensitive, faster, and cost-effective than conventional Sanger sequencing (Gloor et al., 2010; Metzker, 2010). Previous studies have demonstrated the application of NGS techniques, such as metagenomics or shotgun sequencing, for comprehensive microbiome analysis allowing for a deeper understanding of microbial communities and their associations with various factors in NHP species (Amato et al., 2015; Cui et al., 2019; Koo et al., 2019; Pafčo et al., 2019; Sawaswong et al., 2019, 2020, 2021, 2023; Sun et al., 2016). Since the parallel-type short-read sequencer (such as Illumina) could not produce reads of the entire 16S rRNA gene (Ravi et al., 2018), thus several portions needed to be selected for sequencing resulting in taxonomic ambiguity. The advent of new sequencing platforms has overcome these technical restrictions, particularly impacting read length. A prime example was the MinION™ sequencer from Oxford Nanopore Technologies, which can produce long sequences with no theoretical read length limit (Leggett & Clark, 2017). MinION™ sequencing targets the complete 16S rRNA gene, enabling more accurate and sensitive bacterial identification. Furthermore, MinION™ generated sequencing data

in real-time, reducing the turnaround time for data processing (Mitsuhashi et al., 2017). In this thesis, MinION sequencing was also employed for the microbiome analysis in long-tailed macaques using a full-length 16S rRNA gene (V1-V9) following Sawaswong et al. (2023). This allowed the identification of core microbiota up to species levels, while the previous technique (Ravi et al., 2018) had limited to genus-level analysis due to the constraints of short-read 16S sequencing conducted on the Illumina platform. The MinION is also a portable sequencing device that can be easily transported to field sites, enabling real-time sequencing as done previously in *Arabidopsis spp*. (Parker et al., 2017), however it has never been used in the wild NHP populations. This portability is especially valuable for studies conducted in remote locations where access to laboratory facilities may be limited.

CHAPTER III

COMPARATIVE ANALYSIS OF GUT MICROBIOTA BETWEEN COMMON *Macaca fascicularis fascicularis* AND BURMESE *M. f. aurea* LONG-TAILED MACAQUES IN DIFFERENT HABITATS

Introduction

The GI tract harbors about 10–100 trillion microorganisms, comprised of bacteria, viruses, fungi, and parasites, and are collectively called gut microbiota (Gill et al., 2006; Whitman et al., 1998). Bacteria comprise more than 99% of the microorganisms in the gut, of which more than 1,000 species have been identified (Qin et al., 2010). In the past two decades, the gut microbiome has gained increasing attention as a crucial component in the host's immune function, physiology, and behavior. Indeed, recent studies have reported that normal gut microorganisms play a crucial role in health maintenance, including dietary metabolism, vitamin synthesis (Kau et al., 2011), and protection against pathogens (Watanabe et al., 2017). In fact, due to the important roles of the gut microbiota in the host's physiology, it is considered the second genome in animals (Zhu et al., 2010). Changes in the normal gut microbiota composition, so-called dysbiosis, have been associated with various pathologic conditions such as inflammatory bowel diseases (Ferreira et al., 2014), irritable bowel syndrome (Kennedy et al., 2014), metabolic diseases including obesity and diabetes mellitus (Baothman et al., 2016), and allergic diseases (Bisgaard et al., 2011).

The composition of the gut microbiota can be influenced by several factors, including host genetics and the environment. Genetic variation can affect the production of certain digestive enzymes, which can alter the types of nutrients available to the gut microbiota. The genetic variation may affect the immune system, which can affect the gut microbiota (Khachatryan et al., 2008). Diet is one of the most important environmental factors that can influence the composition of gut microbiota; however, diet is strongly linked with the habitat type as this can contribute to the availability and so ingestion of different types of foods or associated microbes, which can affect the composition of the gut microbiota (Claesson et al., 2012; Lan et al., 2017).

Macaca fascicularis is a NHP widely distributed throughout Southeast Asia and classified into 10 subspecies based on their geographical localities and morphological characteristics (Fooden, 1995). Among these 10 subspecies, *Mfa* has acquired attention over the past decade due to their stone-tool use behavior during foraging (Malaivijitnond et al., 2007). In southwestern Thailand, *Mfa* lives in close contact with *Mff*; however, *Mff* has never been reported using percussive stone-tools to forage for foods in either their natural habitat (Fooden, 1995; Malaivijitnond et al., 2011), or in captivity upon training (Bandini & Tennie, 2018). Besides, the hybrids between the *Mfa* and *Mff* were also reported using stone-tools to forage for encased foods in Koram Island, southern Thailand (Lydia V. Luncz et al., 2017; Tan, 2017).

The genetic characteristics, based on mtDNA, Y chromosome *SRY* and *TSPY* genes, whole genome sequences, and autosomal SNPs, are very distinctive between these two subspecies (Bunlungsup et al., 2016; Matsudaira et al., 2018; Osada et al., 2021; Phadphon et al., 2022). Thus, it has been hypothesized that the different

genetics might be one of the factors that led to the emergence and development of stone-tool use in *Mfa* and *Mfa* x *Mff* hybrids but not in *Mff* (Gumert et al., 2019; Reeves et al., 2023). With respect to stone-tool use behavior was found only in *Mfa* and *Mfa* x *Mff* hybrids, it has also been proposed that it might be due to the differences in their habitat types (environment), which in turn could reflect the different types of food availability in their natural habitats. For *Mff*, mainly live on the mainland or fringes of the mainland, such as riverbanks and mangrove forests, while *Mfa* is particularly found on islands (Bunlungsup et al., 2016; Fooden, 1995; Gumert et al., 2009; Luncz et al., 2019). Therefore, it was hypothesized that there might be differences in bacterial microbiomes between the two subspecies of *M. fascicularis* that would reflect the type of habitat and their food types in association with the performance of their stone-tool use behaviors. To date, gut microbiota profiling of *Mff* has been reported, while that of *Mfa* has not yet been carried out (Sawaswong et al., 2019, 2020, 2021, 2023).

Thus, this study aimed to investigate and compare the gut microbiota within the same subspecies of *M. fascicularis* that live in two different habitat types (island and mangrove forest) and between the two different subspecies of *M. fascicularis* (*Mfa* and *Mff*) that live in the same habitat types. This is to understand if the habitat types and/or the diet influence their gut microbiota. Finally, these findings could also highlight the beneficial importance of the complex bacterial microbiota of these macaques that should be pursued in further microbiome research in other species of NHPs and humans.

Methods

Permit and Ethical note

The permits for research and sample collection in the four populations of free-ranging long-tailed macaques sampled in this study in Thailand were approved by the Department of National Parks, Wildlife, and Plant Conservation of Thailand. The Institutional Animal Care and Use Committee (IACUC) of the National Primate Research Center of Thailand-Chulalongkorn University (NPRCT-CU) approved the study's experimental protocols (Protocol Review no. 2075007). The research adhered to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of NHPs. All methods were performed following the relevant guidelines and regulations.

Study sites and consumed food items

To identify the effect of host genetics, diet and habitat type on gut microbiome, two subspecies of free-ranging *M. fascicularis* (*Mff* and *Mfa*) at two habitat types (island and mangrove forest), giving a total of four populations, in Thailand were selected for this study (Table 3.1). The subspecies were identified based on their geographical distribution and morphological characteristics (Bunlungsup et al., 2016; Fooden, 1995; Phadphon et al., 2022). Information on the food type that the monkeys consumed was from direct observation of foraging animals and consumed foods or when the animals finished eating and left the remaining food item(s). Food items were identified and photographed using a Nikon COOLPIX W300 (Nikon, Japan).

Table 3. 1. Subspecies, code, location, geographical coordinate, habitat types, and date of specimen collection of the wild *Mff* and *Mfa* populations in this study.

Subspecies	**Code**	**Location**	**GPS** (N, E)	**Habitat**	**Specimen collection**
Mff	KPE	Koh Ped, Chonburi	12°45′, 100°50′	Island	12 – 16 July, 2022
	BTB	Bang Ta Boon, Phetchaburi	13°15′, 99°56′	Mangrove	17 – 21 July, 2022
Mfa	MFRC	Mangrove Forest Research Center, Ranong	9°52′, 98°36′	Mangrove	26 – 30 July, 2022
	PNY	Piak Nam Yai, Ranong	9°35′, 98°28′	Island	31 July – 6 Aug, 2022

Fecal specimen collection

A total of 120 freshly defecated specimens (n = 30 for each population) were non-invasively collected using the fecal swab method in their natural habitats (Fig. 3.1). In each location, the survey was conducted over at least five consecutive days, at 7:00 AM – 4:00 PM (Table 3.1). To avoid contamination with the soil microbiome, the fecal samples were collected from the inner part using cotton swabs (Citoswab, China). Samples were preserved in 2 mL of DNA/RNA shield (Zymo Research, USA) for viral inactivation and nucleic acid stabilization. To avoid double collection, the physical characteristics (i.e., color, texture, and shape) of each fecal specimen were recorded.

Figure 3. 1. Feces collection from freshly defecated specimens using fecal swab method.

DNA extraction

DNA was extracted using the ZymoBIOMICS™ DNA Miniprep kit (Zymo Research, USA). Briefly, 750 µL of fecal suspension were lysed in a ZR BashingBead™ lysis tube using TissueLyser LT (Qiagen, Germany) at 50 Hz for 3 minutes. The cell lysate was then extracted following the manufacturer's instructions. The concentration of DNA was determined using $A_{260/280\ nm}$ by NanoPhotometer® C40 (Implen, Germany).

PCR amplification and sequencing on MinION™

The full-length bacterial 16S rRNA gene, *ca*. 1,500-bp size, was amplified based on PCR with the specific primers; 16S-V1F 5′-TTTCTGTTGGTGCTGATATTGCAGRGTTYG-ATYMTGGCTCAG-3' and 16S-V9R 5′-ACTTGCCTGTCGCTCTATCTTCCGGYTACC-TTGTTACGACTT-3′

(Matsuo et al., 2021). The 10 µL PCR reaction mixture consisted of 5 µL of 2× UltraHiFi mix (Tiangen, China), 2 µL of PCR Enhancer (Tiangen, China), 0.25 µM each of forward and reverse primers, 1.5 µL of ddH_2O, and 1 µL of the nucleic acid template. The PCR was thermal cycled at 94°C for 2 min, followed by 25 cycles of 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 45 s, and then a final 68 °C for 5 min. The amplicons were barcoded by a 5-cycle PCR using the barcode primers based on the PCR Barcoding Expansion 1-96 (EXP-PBC096) kit (Oxford Nanopore Technologies, UK). The barcoded libraries were enriched using a QIAquick® PCR Purification kit (QIAGEN, Germany) following the manufacturer's instructions. The enriched libraries were quantified by Quant-iT™ dsDNA HS Assay kit using Qubit 4 fluorometer (Invitrogen, USA) then equimolarly pooled for multiplexing. The pooled library was enriched using 0.5× Agencourt AMPure XP beads (Beckman Coulter, USA). Afterwards, the library was subjected to end repair and adaptor ligation steps using Ligation Sequencing Kit (SQK-LSK114). Finally, the library was loaded onto the R10.4.1 flow cell and sequenced on a MinION™ Mk1C sequencer (Oxford Nanopore Technologies, UK).

Data analysis

The FASTQ files were generated from the FAST5 data based on a super-accuracy model with a minimum acceptability quality score (Q > 10) using the Guppy basecaller software v6.0.7 (Oxford Nanopore Technologies, UK) (Wick et al., 2019), while MinIONQC was used for the evaluation of the quality of the reads (Lanfear et al., 2019). Porechop v0.2.4 software (https://github.com/rrwick/Porechop) was used for adaptor-trimming and demultiplexing of FASTQ sequences, while NanoCLUST

was used for clustering, polishing, and taxonomically classifying the filtered reads, based on the size of the sequences for the V1–V9 region of 16S rRNA gene sequences from the Ribosomal Database Project (RDP) database (Cole et al., 2003; Rodríguez-Pérez et al., 2021). The files were converted into QIIME (Quantitative insight into microbial ecology) format and the QIIME2 toolkit v2021.2 was used for the calculation of the alpha diversity using Chao1 and Shannon indices, and the beta diversity by Bray-Curtis cluster analysis (Bolyen et al., 2019). The MicrobiomeAnalyst was used for the visualization of normalized data (Chong et al., 2020). The Galaxy server was used for the differential abundance analysis of gut microbiota using linear discriminant analysis Effect Size (LEfSe) with $P < 0.05$ and a linear discriminant analysis (LDA) score > 2 (Segata et al., 2011). A Neighbor-Joining (NJ) (Saitou & Nei, 1987) phylogenetic tree using the Tamura-Nei model (Tamura & Nei, 1993) was constructed from the selected taxa based on the LEfSe analysis. The DNA sequences of selected bacterial taxa were accessed from GenBank. The evolutionary analysis was conducted in MEGA X (Kumar et al., 2018). Statistical analysis was conducted using non-parametric tests, specifically the Mann-Whitney U and Kruskal-Wallis tests, due to the non-normal distribution of the data.

Results

Food items consumed by *Mff* and *Mfa*

During the field surveys and fecal specimen collections of *Mff* on 12 – 21 July 2022, the *Mff* populations in Koh Ped (KPE) island and Bang Ta Boon (BTB) mangrove forest were frequently provisioned with foods (mostly as fresh fruits, such as banana, watermelon, guava, and pineapple) by tourists and local people. KPE is a

small island (also known as Monkey Island) situated in Chonburi province and is one of the favorite hotspots for tourists in eastern Thailand. Tourist boats and yachts had regularly visited this island, particularly before the COVID-19 pandemic, and stayed for a long time because of the scenic view. As a result, the tourists have access to the KPE *Mff* and feed them. The BTB *Mff* population inhabited a mangrove forest in Phetchaburi province, southern Thailand. Local people often visited the location and fed the monkeys.

The *Mfa* habitat sites were surveyed, and fecal specimens were collected between 26 July – 06 August 2022. The two *Mfa* populations lived in the Mangrove Forest Research Center (MFRC) and Piak Nam Yai (PNY) Island, situated in Ranong province, along Andaman Sea Coast, southwestern Thailand. The MFRC was under the authority of the Department of Marine and Coastal Resources, while the PNY population was under the authority of the Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment. For more than 3 years, these two locations were closed to visitors because of the COVID-19 situation. These *Mfa* populations became less habituated to humans and were roaming freely for natural foods.

Thus, the food items consumed by *Mff* and *Mfa* were categorized into natural foods, including marine invertebrates and plants, and foods provisioned by humans (Fig. 3.2). A detailed list of the type of food consumed by these populations is available in Table 3.2 – 3.5.

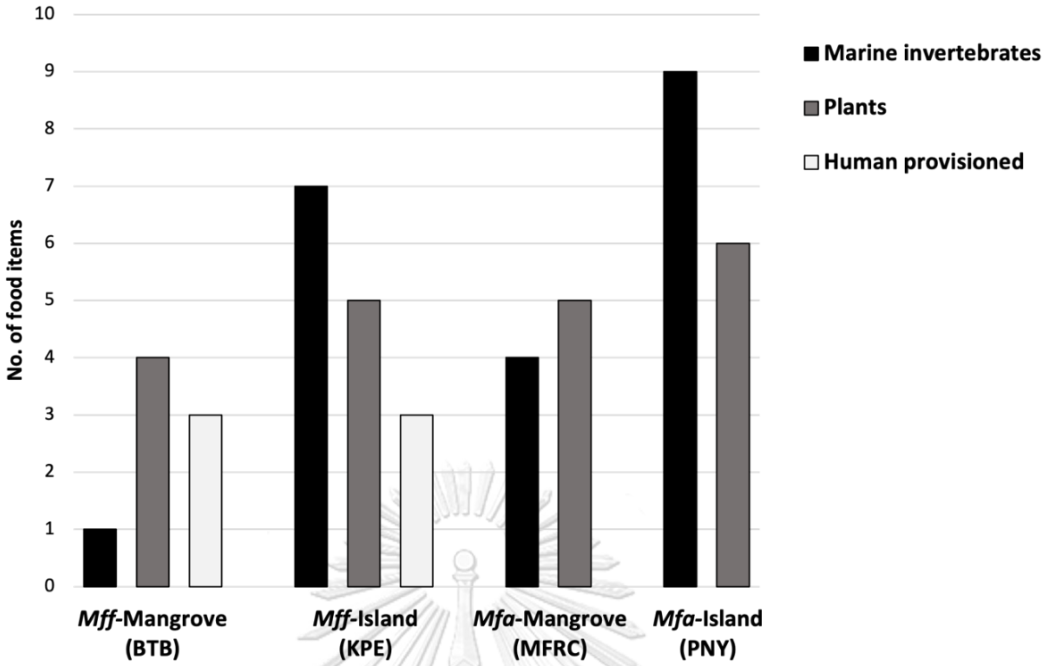

Figure 3. 2. The total number of food items (per visit) consumed by *Mff* and *Mfa* living in a mangrove forest or on an island. The black, grey, and white columns indicate marine invertebrates, plants, and human-provisioned foods, respectively.

Table 3. 2. A list of food types consumed by *Mff* at BTB mangrove forest.

Natural foods							
No	**Food group**	**Kingdom**	**Phylum**	**Class**	**Genera**	**Specie**	**Vernacular names**
1.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Saccostrea*	*cucullata*	Rock oyster
2.	Plant	Plantae	Tracheophyta	Magnoliopsida	*Avicennia*	*alba*	Leaf
3.	Plant	Plantae	Tracheophyta	Magnoliopsida	*Avicennia*	*alba*	Fruit
4.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Amaranthus*	*viridis*	Slender amaranth
5.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Achyranthes*	*aspera*	Leaf
Human food provisioning							
1.	Banana						
2.	Guava						
3.	Watermelon						

Table 3. 3. A list of food types consumed by *Mff* at KPE Island.

Natural foods							
No	**Food group**	**Kingdom**	**Phylum**	**Class**	**Genera**	**Specie**	**Vernacular names**
1.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Saccostrea*	*forskali*	Rock oyster
2.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Pinna*	*bicolor*	Bicolored pen shell
3.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Anadara*	*granosa*	Blood clam
4.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Anadara*	*inaequivalvis*	Hairy cockle
5.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Gafrarium*	-	
6.	Marine invertebrate	Animalia	Arthropoda	Malacostraca		-	Crab
7.	Marine invertebrate	Animalia	Chordata	-	-	-	Fish
8.	Plant	Plantae	Spermatophyta	Monocotyledoneae	*Zea*	*mays*	Corn
9.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Coccinia*	*grandis*	Scarlet gourd
10.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Colubrina*	*asiatica*	Latherleaf
11.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Sida*	*acuta*	Sida
12.	Plant	Plantae	-	-	-	-	Leaf
Human food provisioning							
1.	Watermelon						
2.	Banana						
3.	Pineapple						

Table 3. 4. A list of food types consumed by *Mfa* at MFRC mangrove forest.

Natural foods							
No	**Food group**	**Kingdom**	**Phylum**	**Class**	**Genera**	**Specie**	**Vernacular names**
1.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Saccostrea*	*cucullata*	Rock oyster
2.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Geloina*	-	-
3.	Marine invertebrate	Animalia	Arthropoda	Malacostraca	*Grapsus*	*albolineatus*	Shore crab
4.	Marine invertebrate	Animalia	Arthropoda	Malacostraca	-	-	Crab
5.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Rhizophora*	*mucronata*	loop-root mangrove
6.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Excoecaria*	*agallocha*	Milky mangrove
7.	Plant	Plantae	Tracheophyta	Magnoliopsida	*Xylocarpus*	*moluccensis*	Nyireh batu
8.	Plant	Plantae	Tracheophyta	Magnoliopsida	*Xylocarpus*	*granatum*	Cannonball mangrove
9.	Plant	Plantae	Tracheophyta	Magnoliopsida	*Ceriops*	*tagal*	Spurred mangrove

Table 3. 5. A list of food types consumed by *Mfa* at PNY Island.

Natural foods							
No	**Food group**	**Kingdom**	**Phylum**	**Class**	**Genera**	**Specie**	**Vernacular names**
1.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Saccostrea*	*cucullata*	Rock oyster
2.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Barbatia*	-	-
3.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Ruditapes*	-	-
4.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Gafrarium*	*divaricatum*	forked Venus
5.	Marine invertebrate	Animalia	Mollusca	Bivalvia	*Asaphis*	*violascens*	Pacific asaphis
6.	Marine invertebrate	Animalia	Mollusca	Gastropoda	*Nerita*	*chamaeleon*	Chameleon nerite
7.	Marine invertebrate	Animalia	Mollusca	Gastropoda	*Pugilina*	*cochlidium*	Crown conch
8.	Marine invertebrate	Animalia	Mollusca	Gastropoda	*Laevistrombus*	*canarium*	Dog conch
9.	Marine invertebrate	Animalia	Arthropoda	Malacostraca	*Thalamita*	-	Swimming crab
10.	Plant	Plantae	Magnoliophyta	Magnoliopsida	*Terminalia*	*catappa*	Sea almond fruiting
11.	Plant	Plantae	Magnoliophyta	Liliopsida	*Pandanus*	*tectorius*	Seashore pandan
12.	Plant	Plantae	Spermatophyta	Monocotyledoneae	*Cocos*	*nucifera*	Coconut
13.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Parkia*	*speciosa*	Bitter bean
14.	Plant	Plantae	Spermatophyta	Dicotyledonae	*Rhizophora*	*mucronata*	loop-root mangrove
15.	Plant	Plantae	-	-	-	-	Fiber

Nanopore sequencing of bacterial 16S rRNA gene

The full-length bacterial 16S rRNA gene from 120 fecal samples of long-tailed macaques was successfully sequenced using high throughput nanopore sequencing. In total, 2,444,551 sequencing reads were obtained from 120 samples, with an average read per sample of 20,371 (Table 3.6). The average classified reads were 18,091 per sample. According to the rarefaction analysis, which is primarily used to determine if the richness of the samples has been fully observed or sequenced. The rarefaction plot (Fig. 3.3) showed that at the beginning of the curve, the slope was steep, indicating that as sequencing depth increases, the observed microbial diversity also increases rapidly. When the rarefaction curve entered the plateau phase, it suggested that the sequencing depth was sufficient to capture the majority of the microbial diversity in the samples that had been sequenced. This indicated that further sequencing is unlikely to reveal significant additional bacterial diversity.

Table 3. 6. Summary of the sequencing and reads classification (mean ± SD) in each population of *Mff* and *Mfa* in the two respective habitat types.

Subspecies	Habitat type	Population	Raw read	Retained reads
Mff	Mangrove	BTB	21,413 ± 6,693	18,470 ± 5,833
	Island	KPE	19,489 ± 6,298	17,180 ± 5,660
Mfa	Mangrove	MFRC	18,402 ± 6,053	16,638 ± 5,552
	Island	PNY	22,179 ± 6,294	20,077 ± 5,745

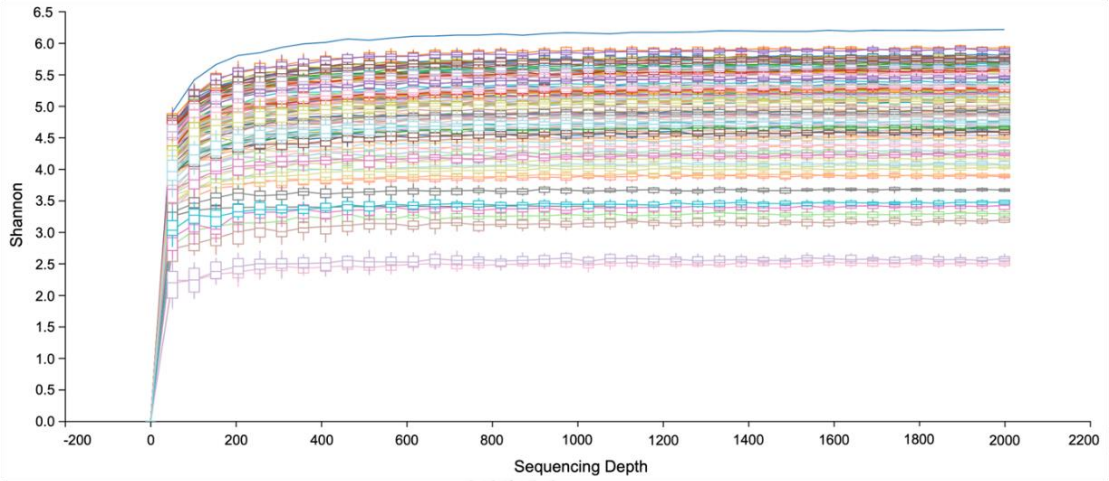

Figure 3. 3. Rarefaction analysis showed that an adequate sequencing depth was obtained for estimating the diversity of all samples.

Bacterial diversity in the gut microbiome in *Mff* and *Mfa*

Bacterial alpha diversity (level of diversity within individual samples) comparisons between *Mff* and *Mfa* in the respective mangrove and island populations were evaluated based on the Chao1 index (Fig. 3.4A), while the richness and evenness of bacterial operational taxonomic units (OTUs) were determined using the Shannon diversity index (Fig. 3.4B). Statistical comparisons of indices between groups were carried out using a Kruskal-Wallis test, accepting significance at the $P < 0.05$ level.

The Chao1 index and Shannon's diversity between different habitat types of the *M. fascicularis* subspecies were compared. The Chao1 index of the KPE-*Mff* population on the island had a significantly higher OTU richness ($P = 0.0021$) than the BTB-*Mff* population in the mangrove forest. Likewise, Shannon's diversity was noticeably and significantly higher ($P = 0.0002$) for the KPE-*Mff* island population than the BTB-*Mff* mangrove population. Similarly, the PNY-*Mfa* population living on

the island showed a significantly higher OTU richness (*P = 0.0021*) and Shannon's diversity index (*P = 0.0332*) than the MFRC-*Mfa* mangrove population. Overall, the alpha diversity of *Mff* was significantly higher than that for the *Mfa* populations in both habitat types.

To further examine the differences between the samples, beta diversity (level of diversity or dissimilarity between samples) analyses were performed using the Bray-Curtis cluster analysis index to compare the microbial community compositions between *Mff* and *Mfa* in mangrove and island populations. The beta diversity (Fig. 3.4C) between *Mff* and *Mfa* in different habitat types (mangrove and island) were significantly different (*P = 0.001*, permutational multivariate analysis of variance [PERMANOVA]). However, the MFRC-*Mfa* mangrove population had a significant divergence from the other populations.

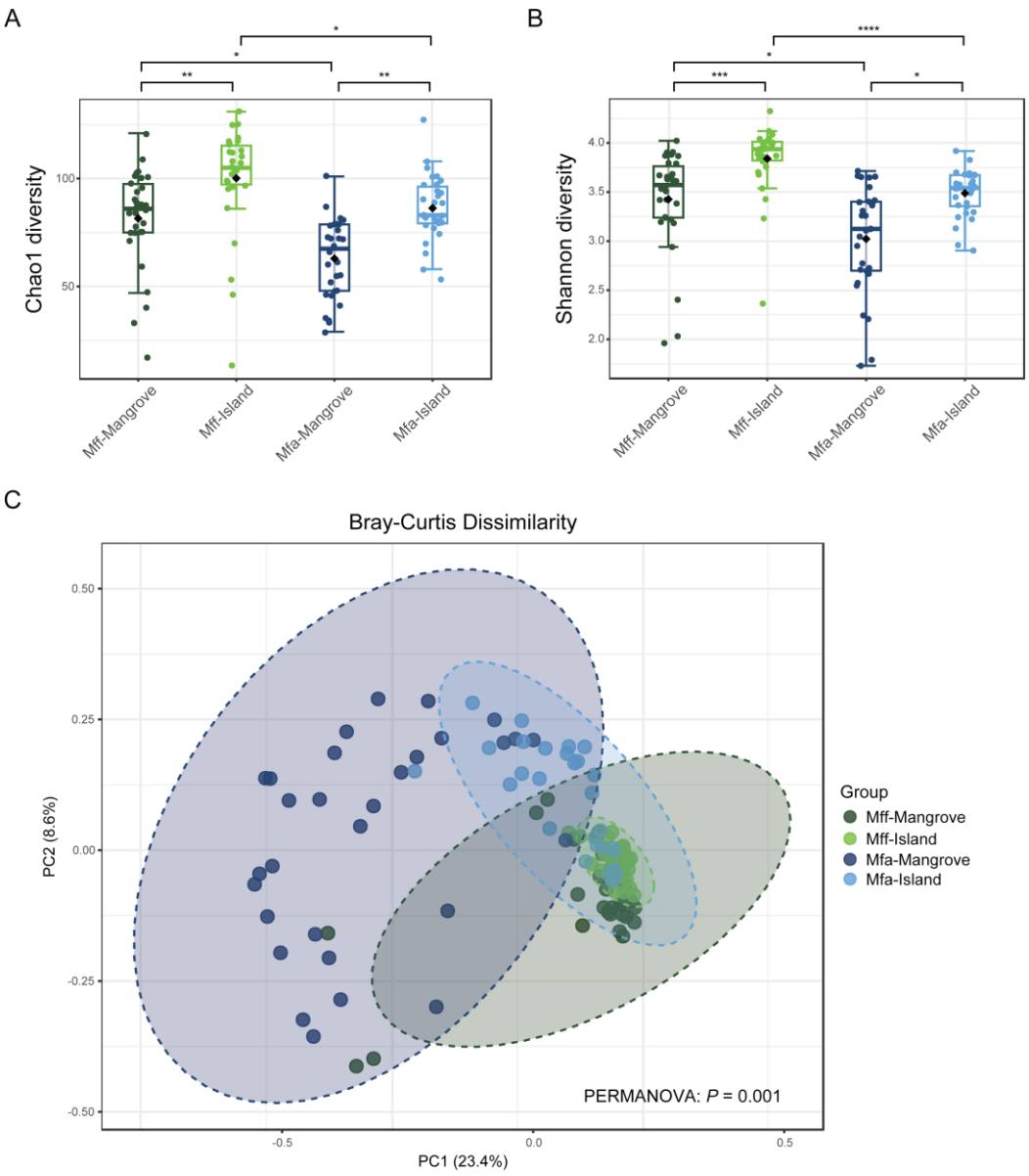

Figure 3. 4. Gut microbiome bacterial diversity in *Mff* and *Mfa* living in mangrove and island habitats. Alpha diversity was compared by the **A)** Chao1 and **B)** Shannon indices, while **C)** Beta diversity was measured by principal coordinate analysis (PCoA) using Bray-Curtis distance. Alpha diversity was statistically tested by the Kruskal-Wallis test *(*P < 0.05, **P < 0.01, ***P < 0.001,* and *****P < 0.0001)*, while PERMANOVA was used for the beta diversity *(P = 0.001)*.

Taxonomic composition of the gut microbiota in *Mff* and *Mfa* at mangrove and island habitats

Comparison of the bacterial species between the same macaque subspecies (*Mff* and *Mfa*) in the different habitat types (island or mangrove) was examined by Mann-Whitney U tests ($P < 0.05$). Firmicutes were the most dominant bacterial phylum among the mangrove and island *Mff* populations (Fig. 3.5, upper panel) at mean ± SD of 57.6 ± 14.6% and 57.3 ± 5.9%, respectively. The Bacteroidetes accounted for 24.0 ± 10.2% and 28.9 ± 6.8% in the *Mff* mangrove and island populations, respectively, making it the second most abundant phylum. However, the relative abundance of Firmicutes and Bacteroidetes was not significantly different between the *Mff* mangrove and island populations (4.7 ± 8.1 for mangrove and 2.1 ± 1.9 for island population; Mann – Whitney U test; $P < 0.05$). Proteobacteria, which comprised 8.7 ± 18.0% and 4.4 ± 2.0% of the bacteriome in the *Mff* mangrove and island populations, respectively, was the third most dominant phylum. Verrucomicrobia, Spirochaetes, Actinobacteria, and Lentisphaerae were among the least abundant phyla in the *Mff* mangrove and island populations.

Similarly, Firmicutes was the most dominant phylum in the *Mfa* mangrove (74.7 ± 27.2%) and island (64.9 ± 17.9%) populations, while Bacteroidetes was the second most abundant phylum in both *Mfa* populations (5.4 ± 10.7% and 20.6 ± 13.2% for the mangrove and island populations, respectively). In contrast to the *Mff* populations, the relative abundance of Firmicutes to Bacteroidetes ratio in the PNY-*Mfa* island population (8.9 ± 12.4) was significantly lower than the MFRC-*Mfa* mangrove population (68.3 ± 82.5) (Mann – Whitney U test; $P < 0.001$), which was aligned with the higher abundant level of the Bacteroidetes in the PNY-*Mfa* island

population. The Proteobacteria (14.0 ± 25.8%) and Verrucomicrobia (6.7 ± 6.1%) were the third most abundant phyla in the *Mfa* mangrove and island population, respectively.

The top 10 most dominant genera in the bacterial communities of both macaque subspecies in mangrove and island populations were identified and shown in Fig. 3.5, middle panel. The most dominant bacterial genus in *Mff* was *Oscillibacter* at 13.3 ± 6.5% and 12.2 ± 3.3% in the mangrove and island population, respectively. The other predominant bacteria in the bacterial microbiome of *Mff* were *Prevotella*, *Clostridium sensu stricto*, *Clostridium XlVa*, *Faecalibacterium* and *Intestinimonas*. The proportions of these bacteria varied across samples and differed between the *Mff* mangrove and island populations. *Oscillibacter* was also the most dominant bacterial genus in the fecal microbiome of the *Mfa* island population (17.8 ± 7.6%) and was higher than that in the *Mfa* mangrove population (4.9 ± 4.5%). In contrast, *Clostridium sensu stricto* was the most predominant bacterial genus in the *Mfa* mangrove population (23.1 ± 22.9%) and had significantly higher abundance ($P < 0.0001$) than the *Mfa* island population (4.9 ± 7.4%).

At the species level, *Oscillibacter valericigenes* was the most dominant bacterial species in *Mff* at 13.38 ± 6.56% and 12.20 ± 3.36% in the mangrove and island populations, respectively (Fig. 3.5, lower panel). The other less dominant bacterial species were *Prevotella copri*, *Intestinimonas butyriciproducens*, and *Faecalibacterium prausnitzii*; however, their abundance varied between populations. In contrast, *Clostridium sardiniense* was the most predominant bacterial species in the *Mfa* mangrove population (14.0 ± 15.5%) with a significantly higher abundance ($P < 0.00001$) than in the *Mfa* island population (0.03 ± 0.1%).

Comparison of the bacterial species between the different macaque subspecies (*Mff* and *Mfa*) in the same habitat types (island or mangrove) was examined by Mann-Whitney U tests ($P < 0.05$). The results revealed that the Firmicutes and Bacteroidetes were the two most abundant phyla in the *Mfa* and *Mff* island populations; however, Firmicutes were not significantly different between them, whereas Bacteroidetes were significantly higher in the *Mff* (28.9 ± 6.8%) than in the *Mfa* (20.6 ± 13.2%) island populations. Similarly, the mangrove population of *Mfa* showed a significantly higher abundance of Firmicutes (74.7 ± 27.2%) and a lower abundance of Bacteroidetes (5.4 ± 10.7%) than the *Mff* mangrove population. The taxonomic composition at a genera level showed that the *Mfa* island population (17.8 ± 12.2%) had a significantly higher abundance of *Oscillibacter* than the *Mff* island population (12.2 ± 3.3%), while *Clostridium sensu stricto* was significantly higher in the *Mfa* mangrove population (23.1 ± 22.9%) than in the *Mff* mangrove population (5.8 ± 11.1%). Further comparison at the species level revealed that *Oscillibacter valericigenes* was significantly more abundant in the *Mfa* island population (17.8 ± 7.6%) than in the *Mff* island population (12.2 ± 3.3%), while the *Mfa* mangrove population (14.0 ± 15.5%) had a significantly higher abundance of *Clostridium sardiniense* than the *Mff* mangrove population (0.4 ± 1.6%).

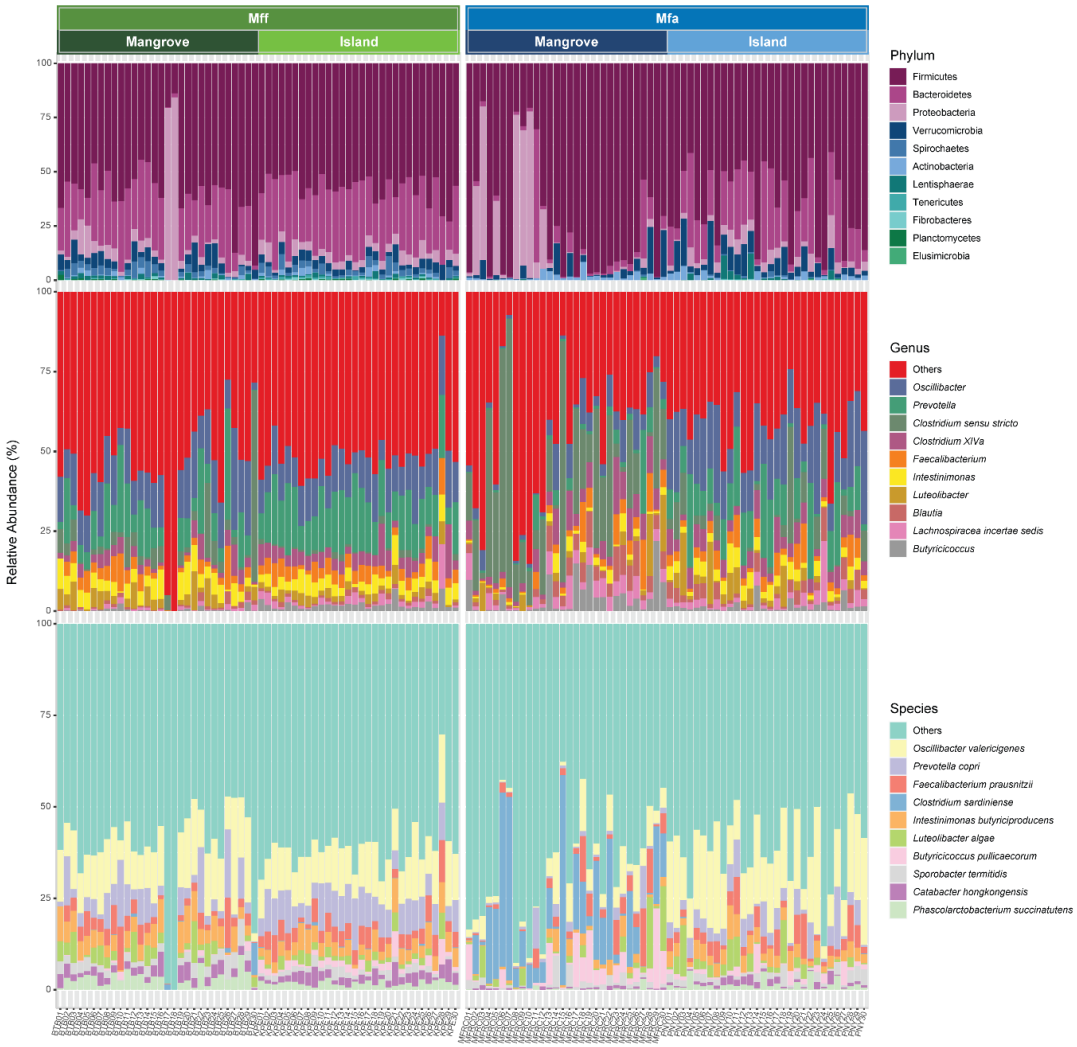

Figure 3. 5. The relative abundance (%; stacked bars) of gut microbiome in *Mff* and *Mfa* living in mangrove and island habitats. The bacterial phyla, genera, and species levels are presented in upper, middle, and lower panels.

Differential abundance of gut bacteria between *Mff* and *Mfa* in different habitat types

The taxonomic abundance of the gut bacterial microbiota of *Mff* and *Mfa* living in the mangrove forest and on the island were compared further using the LEfSe analysis (LDA score>2, $P<0.05$) (Segata et al., 2011), as shown in Fig. 3.6. Differences in the gut bacterial microbiota between the *Mff* and *Mfa* populations in the different habitat types of mangrove forest and island were identified. *Porphyromonadaceae, Phascolarctobacterium succinatutens, Acidaminococcaceae,* and *Prevotella fusca* were the most enriched taxa in the BTB-*Mff* mangrove population, while the KPE-*Mff* island population had a greater number of significantly enriched taxa, including *Tannerella forsythia, Bdellovibrionaceae, Rikenella microfusus, Barnesiella viscericola, Ethanoligenens harbinense, Olivibacter sitiensis,* and *Fibrobacter intestinalis.* In contrast, the MFRC-*Mfa* mangrove population was enriched in *Lachnospiraceae incertae sedis, Clostridium saccharolyticum,* and *Eubacterium hallii*. Moreover, *Haloferula helveola* and *Bacteroides fluxus* were abundant in the PNY-*Mfa* island population. These results indicate the significant differences in the compositional abundance of gut microbiota between *Mff* and *Mfa* in the mangrove and island populations.

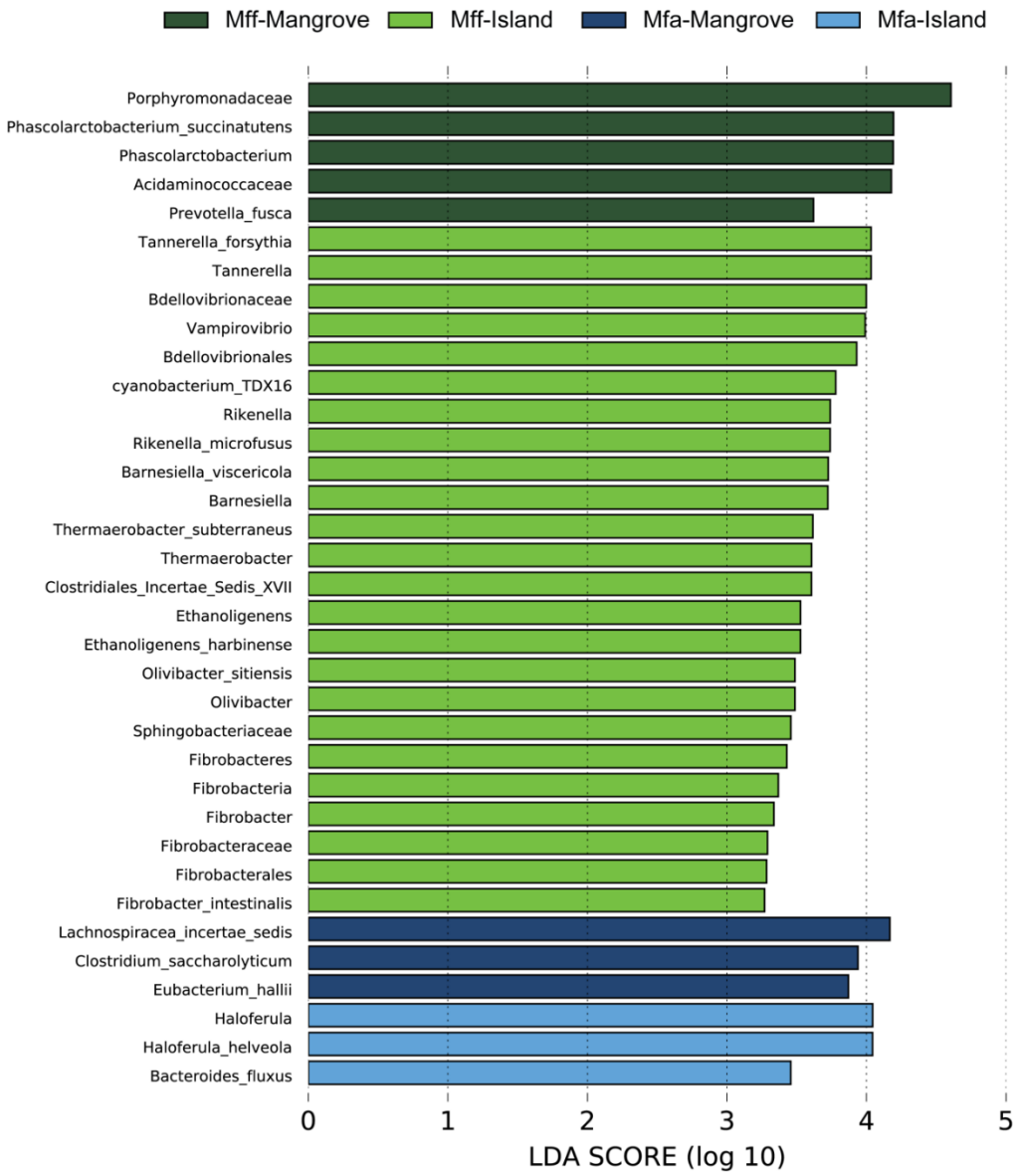

Figure 3. 6. Differential abundance analysis by Linear discriminant analysis Effect Size (LEfSe) of the gut bacterial microbiome of *Mff* and *Mfa* living in a mangrove forest and on the island. The bar plots indicated the differentially abundant bacterial microbiota at different taxonomic ranks. The LDA score shows the effect size and ranking of each differentially abundant taxon (LDA score > 2, $P < 0.05$).

Phylogenetic diversity of microbes

Based on the LEfSe analysis mentioned above, only bacterial taxa that could be identified into species level in each population were selected for the construction of the NJ phylogenetic tree. Thus, it might not represent the most predominant species. Accession numbers of 13 bacterial sequences are presented in Table 3.7. The phylogenetic tree was divided into two major clades, Bacteroidetes and Firmicutes/Verrucomicrobia /Fibrobacteres (Fig. 3.7). All populations, except MFRC-*Mfa*, had bacterial taxa in both clades. The KPE-*Mff* island population had the highest diversity of enriched taxa belonging to Bacteroidetes (*R. microfusus*, *T. forsythia* and *B. viscericola*), Firmicutes (*F. intestinalis* and *O. sitiensis*), and Fibrobacteres (*E. harbinanense* and *T. subterraneus*), while the bacterial taxa of BTB-*Mff* mangrove population belonged to Bacteroidetes (*P. fusca*) and Firmicutes (*P. succinatutens*). On the other hand, the microbe in *Mfa* populations was less diverse than the *Mff*. The PNY-*Mfa* island population had Bacteroidetes (*B. fluxus*) and Verrucomicrobia (*H. helveola*) taxa, and the MFRC-*Mfa* mangrove population, showed the two enriched taxa, *C. saccharolyticum* and *E. hallii*, belonging to the Firmicutes, which was closely related.

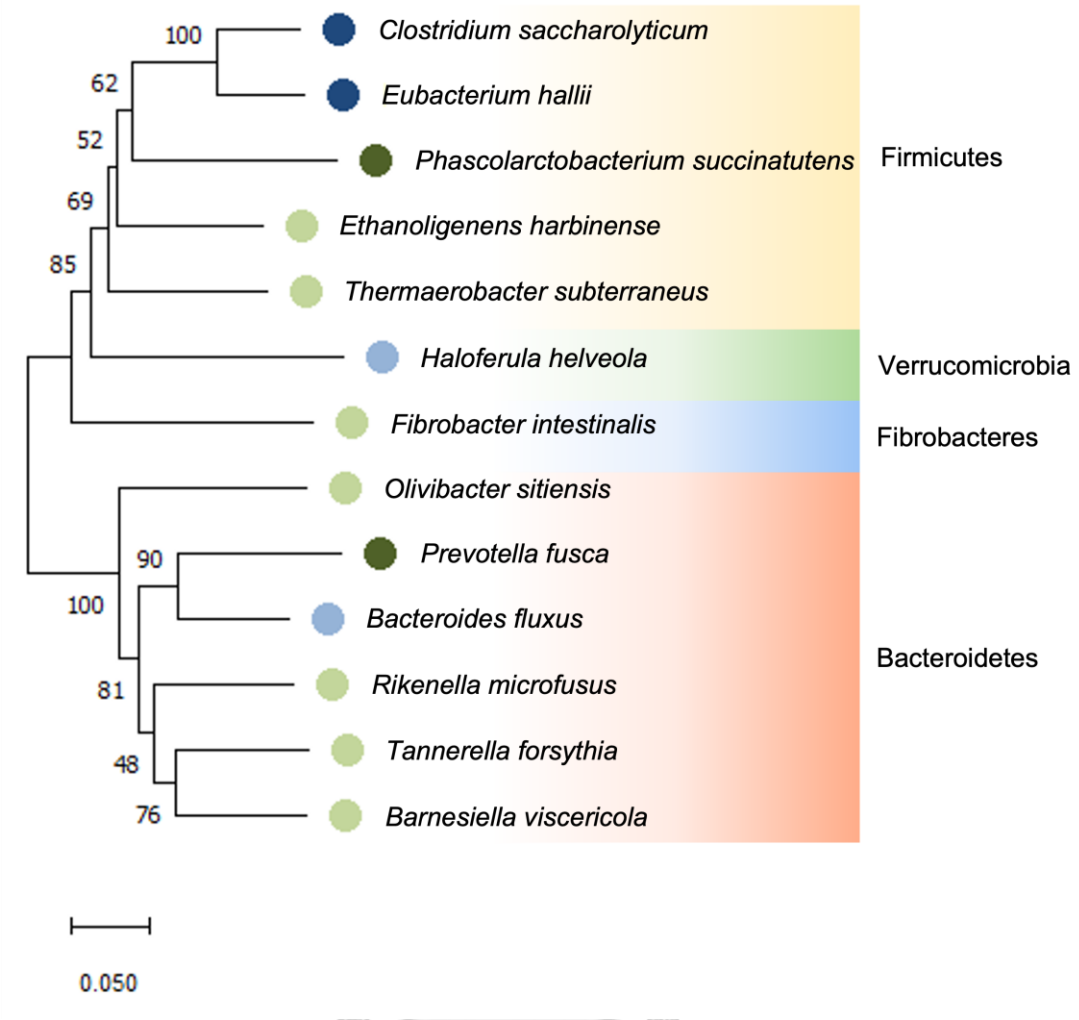

Figure 3. 7. NJ phylogenetic tree of bacterial taxa of MFRC-*Mfa* (dark blue dot), PNY-*Mfa* (light blue dot), BTB-*Mff* (dark green dot), and KPE-*Mff* (light green dot) populations.

Table 3. 7. Accession numbers of 13 bacterial sequences used for NJ phylogenetic tree.

S. No.	Bacterial Species	Accession No.
1.	*Clostridium saccharolyticum*	NR_102852.1
2.	*Eubacterium hallii*	NR_118673.1
3.	*Phascolarctobacterium succinatutens*	NR_112902.1
4.	*Ethanoligenens harbinense*	NR_074333.1
5.	*Thermaerobacter subterraneus*	NR_028814.1
6.	*Fibrobacter intestinalis*	NR_042150.1
7.	*Haloferula helveola*	NR_041673.1
8.	*Olivibacter sitiensis*	NR_043805.1
9.	*Rikenella microfusus*	NR_025910.1
10.	*Barnesiella viscericola*	NR_121773.2
11.	*Tannerella forsythia*	NR_040839.1
12.	*Prevotella fusca*	NR_114304.1
13.	*Bacteroides fluxus*	NR_112894.1

Discussion

Comparative analysis of the gut bacterial microbiota between different subspecies of *M. fascicularis* (*Mff* and *Mfa*) living in different habitat types (i.e., mangrove and island) revealed the potential influence of different host genetic, environments and diets on their gut bacterial composition. Overall, the alpha diversity of gut bacteria in *Mff* was significantly higher than in the *Mfa* populations, which probably reflected the higher enrichment of bacterial species in *Mff* populations (BTB

and KPE), such as *Oscillibacter valericigenes*, *Prevotella copri*, *Faecalibacterium prausnitzii*, and *Intestinimonas butyriciproducens*, caused by human provisioning of alternative foods. A previous study in rhesus macaques also indicated that a population that was provided with foods by humans had a higher microbial richness than a wild population that freely foraged for natural foods (Chen et al., 2020). Generally, the gut microbial diversity is often higher in wild animals than in captive animals due to the composition of their complex diet (McKenzie et al., 2017; Nelson et al., 2013; Sawaswong et al., 2023; Sawaswong et al., 2021). One possible explanation for the higher bacterial richness in the *Mff* KPE and BTB populations is that, apart from the natural foods in their natural habitats, these *Mff* could access additional foods regularly provided by humans. These findings suggest that the types of food consumed by the *Mff* played a key role in shaping the gut microbiome in terms of their bacterial composition. However, the effect of host (macaque) genetics, which is very different between *Mff* and *Mfa*, and the habitat types cannot be neglected.

Comparing the bacterial diversity between *Mff* and *Mfa* living in the same habitat type, the BTB-*Mff* mangrove population demonstrated a higher bacterial diversity than the MFRC-*Mfa* mangrove population, and the KPE-*Mff* island population also exhibited a higher bacterial diversity than the PNY-*Mfa* island population. These results implied that while diet (human-provisioned foods) and habitat type are crucial factors influencing the bacteriome, the host genetic likely contributed to the differences in bacterial diversity. A previous study reported that the host genetic influenced bacteriome diversity in four NHP species: *Chlorocebus pygerythrus* (vervet monkey), *Chlorocebus sabaeus* (green monkey), *M. mulatta*

(rhesus macaque), and *M. nemestrina* (southern pig-tailed macaque) that were fed the same diets (Flynn et al., 2023). Thus, it is necessary to explore further the impact of the distinctive host genetic (*Mff* and *Mfa*) on the microbiome composition, for example, the study of microbiome of captive *Mff* and *Mfa* populations housed under the same diet program. Such investigations would provide a more comprehensive understanding of the influence of the host genetic on microbial communities. Regarding different bacterial diversity between each *M. fascicularis* subspecies (*Mff* and *Mfa*) living in different habitat types, the island (PNY-*Mfa* and KPE-*Mff*) populations always had higher bacterial diversity than the mangrove (MFRC-*Mfa* and BTB-*Mff*) populations. Noted, both *Mfa* populations relied only on natural food sources in their respective habitats and both *Mff* populations were additionally provided human-foods. These findings suggested that habitat type also played a prominent role in shaping bacterial diversity. Noted, the KPE-*Mff* population was recently discovered using stone-tools to access the marine (i.e., oysters) foods (see Chapter 4). Thus, the higher number of marine invertebrate foods in KPE-*Mff* than the BTB-*Mff*, acquired using stone-tools, needs to be kept in mind, and an association between microbiome diversity and stone-tool use behavior should also be considered. Besides, the number of marine invertebrate foods in KPE-*Mff* population was comparable to those of the MFRC-*Mfa* and the PNY-*Mfa*.

The lower observed Chao 1 and Shannon alpha diversity of the microbiome in the MFRC-*Mfa* mangrove population might be explained by the high abundance of the single bacterial species of *Clostridium sardiniense*. Previously, it was reported that increased stress, dietary changes, and other external factors were the causes of a lower alpha diversity in the gut microbiome of captive NHPs (Clayton et al., 2016;

Frankel et al., 2019; McKenzie et al., 2017; Tan et al., 2014); however, dysbiosis of the gut microbiome with a lower alpha diversity in wild primates has not yet reported.

The gut microbiota of *Mff* and *Mfa* in this study were mainly composed of two phyla, the Firmicutes and Bacteroidetes, which were most likely similar to that of humans and other NHPs, including other wild and captive Thai *Mff* (Eckburg et al., 2005; Fogel, 2015; Gomez et al., 2015; Sawaswong et al., 2023; Sawaswong et al., 2020; Sawaswong et al., 2019; Sawaswong et al., 2021; Trosvik et al., 2018). Note that the composition of Firmicutes in the MRC-*Mfa* mangrove population was highest among the four examined populations of long-tailed macaques. Thus, the relative abundance of Firmicutes varied between different subspecies and different habitat types; the MFRC-*Mfa* mangrove population had a higher relative abundance than the PNY-*Mfa* island population, and the *Mfa* populations had a higher relative abundance than the other two *Mff* populations. The Firmicute species contains numerous genes encoding enzymes related to energy metabolism, and these bacteria can produce a wide variety of digestive enzymes to decompose various substances, assisting the host in the digestion and absorption of nutrients (Kaakoush, 2015).

According to previous studies, a higher ratio of Firmicutes to Bacteroidetes is associated with higher absorption of dietary energy (Clarke et al., 2013; Turnbaugh et al., 2006). Bacteroidetes species help the host metabolize the proteins and carbohydrates in the diet (Fernando et al., 2010; Lapébie et al., 2019). Taken together, it can suggest that the abundance of Firmicutes and the ratio of Firmicutes to Bacteroidetes are related to the genetic characteristics (leading to a different subspecies of *Mff* and *Mfa*), habitat type (mangrove forests or island), and the foods provisioned by humans (only in *Mff* populations). The higher abundance of Firmicutes

to Bacteroidetes may partially be related to the consumption of the high-energy mollusk foods that were observed to be heavily consumed in the *Mfa* populations in this study. Based on the short time stay and the fact that no individual animals were identified in this study, the data of proportion of food types that animals consumed each day was not collected. It would be better to identify each animal individually, collect data on the proportion of its daily food consumption and analyze the microbiome composition at an individual level. In addition, the abundance of bacteria belonging to the phylum Proteobacteria in the MFRC-*Mfa* mangrove population was significantly higher than in the PNY-*Mfa* island population, which could reflect the effects of habitat type and food items, possibly the mangrove.

At the genus level, the results indicated that the microbiome of long-tailed macaque populations was enriched with *Prevotella*, one of the most predominant genera in the human microbiome. In line with these findings, a previous study also reported that the macaque microbiome exhibited a higher abundance of *Prevotella* than the human microbiome (Chen et al., 2018). The predominance of *Prevotella* was associated with a diet high in carbohydrates and fiber from plant sources (Wu et al., 2011). Similarly, western lowland gorillas (*Gorilla gorilla gorilla*) that consumed an increased number of fruits had a high relative abundance of Prevotellaceae (Hicks et al., 2018).

At the species level, *Oscillibacter valericigenes* was the most dominant bacterial species in the *Mfa* and *Mff* populations except in the MFRC-*Mfa* mangrove population. *O. valericigenes* is a representative bacterium in the Oscillibacter group that can produce valerate (Iino et al., 2007), a SCFA that can replace butyrate as an energy source for colonocytes. This bacterium's abundance showed its potential

relevance to the macaque's health. These results were consistent with a previous study reported a significant abundance of *O. valericigenes* in healthy humans (Mondot et al., 2011). Similarly, *Faecalibacterium prausnitzii* was present in all four populations of long-tailed macaques examined in this study, which is supported by previous studies that *F. prausnitzii* was the dominant butyrate producer of *Clostridium cluster IV*, the most common bacteria in the microbiome of humans, and which exhibited anti-inflammatory effects (Sokol et al., 2008) and enhanced the gut barrier functions (Carlsson et al., 2013). The depletion of *F. prausnitzii* is associated with Chron's disease (Sokol et al., 2008; Sokol et al., 2009). Note that the microbiome of the *Mfa*-MFRC mangrove population was enriched with *Clostridium sardiniense*, a glycolytic cluster I species that uses anaerobic carbohydrate fermentation to produce butyrate (Wang et al., 2005). This species can also promote a more severe infection of *Clostridioides difficile* in mice by modulating the virulence, growth, and colonization of the pathogen (Girinathan et al., 2021).

The differential species abundance analysis by LEfSe revealed differences in the gut microbiota of *Mff* and *Mfa* in mangrove and island populations. Porphyromonadaceae and *P. succinatutens*, which were the most enriched taxa in the BTB-*Mff* mangrove population, have a potential role as adiposity modulators by producing two SCFAs: acetate and propionate (Tavella et al., 2021; Watanabe et al., 2012). One of the most prominent oral human pathogens was *Tannerella forsythia* (Sharma, 2010), detected at a higher abundance in the KPE-*Mff* island population. Periodontitis in humans is strongly associated with the presence of *T. forsythia*, and this species has a significant role in the pathogenicity of the microbiota in subgingival plaques (Lourenço et al., 2014). In the short-time observations during fecal specimen

collection, the KPE-*Mff* island and BTB-*Mff* mangrove populations were seen to be heavily provided with fresh and leftover foods by humans compared to the other *Mfa* populations. In contrast, the PNY-*Mfa* island population, which is not provisioned with food by humans, was enriched in *H. helveola* and *B. fluxus*, the former specie was commonly found in marine environments (Yoon et al., 2008) and was not known to inhabit the human gut in any marked abundance according to the data from the U.S. NIH Human Microbiome Project and the search engine EZ Bio Cloud (https://www.ezbiocloud.net/resources/human_microbiome). This aligned with a previous report indicating that marine invertebrates were the main food source of the PNY-*Mfa* island population (Gumert & Malaivijitnond, 2012). The *B. fluxus* has been isolated from the feces of healthy human individuals (Watanabe et al., 2010); however, one case of its presence in an abdominal infection has been reported (Cobo et al., 2021). The MFRC-*Mfa* mangrove population was enriched with bacterial species from the family Lachnospiraceae, which degrade complex polysaccharides to butyrate that can then be utilized for energy (Biddle et al., 2013). Herbivores are known to have a higher abundance of Lachnospiraceae than omnivores (Furet et al., 2009). These results might reflect the dietary constituents of the MFRC mangrove population, where the macaques also had access to different plant-based dietary sources in their habitat.

The NJ phylogenetic tree constructed based on selected bacterial taxa showed the two major clades, Bacteroidetes and Firmicutes/Verrucomicrobia /Fibrobacteres, of microbiome of *Mff* and *Mfa* in different habitat types. However, only the MFRC-*Mfa* mangrove population showed the predominance of *C. saccharolyticum* and *E. halli* species which belong to Firmicutes subclade, Firmicutes/Verrucomicrobia

/Fibrobacteres clade. These results are also consistent with the higher abundance of Firmicutes in MFRC-*Mfa* mangrove population. The *C. saccharolyticum* was involved in the carbohydrate degradation (Murray, 1986), while *E. halli* can utilize glucose as well as glycan fermentation intermediate acetate, and lactate to form butyrate (Engels et al., 2016). Thus, diet rich in fiber promotes the growth and activity of *C. saccharolyticum* and *E. hallii,* which indicated that the dietary components of the MFRC-*Mfa* mangrove population, who had access to various plant-based food sources in their habitat, may have influenced the observed results. Considering the mangrove habitat, although the BTB-*Mff* mangrove population was enriched in *P. succinatutens,* also belonging to Firmicutes subclade, this bacterial taxon possessed the enzymatic activity to break down complex carbohydrates including succinate (Watanabe et al., 2012). Thus, the BTB-*Mff* could have *P. fusca*, belonging to Bacteroidetes clade, involving in the fiber-utilizing (Chen et al., 2017). Following the KPE-*Mff* island population, which was enriched with several bacterial species, belonging to the phyla Firmicutes, Bacteroidetes, and Fibrobacteres, showed higher bacterial diversity than the BTB-*Mff*. Although the two *Mff* populations were provisioned human-foods, the KPE-*Mff* island population could use stone-tools to access additional marine invertebrates.

In conclusion, this is the first study to report a comparison of the gut microbiomes between different subspecies of *M. fascicularis* (*Mff* and *Mfa*) living in two different habitat types (mangrove and island). The results revealed a significant difference in the gut microbiome associated with the different genetic backgrounds of the animals (comparing the two subspecies of *M. fascicularis* in this case) and the other food types (comparing mangrove forest and island habitats, and human-

provisioned foods). The latter factor could be associated with using stone-tools in foraging for food. It was previously reported that the PNY-*Mfa* island population used percussive stone-tools daily (Gumert et al., 2009; Gumert & Malaivijitnond, 2012; Malaivijitnond et al., 2007), while the MFRC-*Mfa* mangrove population performed only food-pounding behaviors (personal observation). The food-pounding behavior is that the animals used the food (i.e., shell) to pound the food or to pound the stone, while the stone-tool use behavior is to use the stone to pound the food as seen in the PNY-*Mfa* macaques (Phadphon et al., 2022). During the fecal specimen collections, the KPE-*Mff* population, especially adults, was discovered to sporadically use percussive stone-tools for opening oysters (Chapter IV). Finally, the results of this study may provide some insight if the stone-tool use behaviors in association with the diet acquisition in each population of macaques can play a role in the gut microbiome diversity and abundance, which is the next question to explore further.

CHAPTER IV

INFLUENCE OF COVID-19 ON EMERGENCE OF STONE-TOOL USE BEHAVIOR IN A POPULATION OF COMMON LONG-TAILED MACAQUES *Macaca fascicularis fascicularis* IN THAILAND

Introduction

Customary stone-tool use (McGrew, 1998) is not a widespread behavior found throughout the animal kingdom. The use of percussive stone-tools by NHPs in natural settings has been widely studied, and garnered significant attention due to the close evolutionary relationship with technology-dependent humans (Haslam et al., 2009). To date, there are five wild NHPs, including West African chimpanzees (*Pan troglodytes* verus) (Sugiyama & Koman, 1979), bearded capuchins (*Sapajus libidinosus*) (Fragaszy et al., 2004), *Mfa* (Malaivijitnond et al., 2007), yellow-breasted capuchins (*S. xanthosternos*) (Canale et al., 2009), and white-faced capuchins (*Cebus capucinus imitator*) (Barrett et al., 2018) that have been reported to perform stone-tool use behaviors in their natural habitats. Some similarities between NHP stone-tool use and hominin tool evidence suggest similar evolutionary mechanisms behind the development of stone-tool use behaviors. Understanding the circumstances for the emergence of tool use within a population can help us better understand the cognitive and behavioral driving factors that underlie the development of technology and innovation in our lineage.

Macaca fascicularis, commonly known as the long-tailed macaque, is a species of Old-World monkey found throughout Southeast Asia, including Myanmar,

Thailand, Laos, Cambodia, Vietnam, Malaysia, Indonesia, and the Philippines (Fooden, 1995). They were the second most widely distributed macaque species, following the rhesus macaque. The classification system based on geographic distribution and morphological characteristics divided them into ten subspecies (Fooden, 1995). *Mfa* has been the only stone-tool user today, using hammerstones and anvils to open hard-shelled foods such as nuts and oysters (Malaivijitnond et al., 2007; Gumert et al., 2011; Luncz et al., 2017). *Mfa* distributed in Myanmar and based on the phylogenetic analysis; they migrated southeastwardly along the Andaman Sea Coast through the Mergui Archipelago and southwestern Thailand, where they lived in proximity with *Mff* (Fooden, 1995; Bunlungsup et al., 2016; Phadphon et al., 2022). However, the *Mff* subspecies had never been reported to use stones to crack open the encased foods, neither in their natural habitats (Fooden, 1995; Malaivijitnond et al., 2011) nor in captivity upon training (Bandini and Tennie, 2018). Based on the mtDNA, Y chromosome (*SRY* and *TPSY*) genes, whole genome sequences, and autosomal SNPs analyses suggested that the two subspecies are genetically distinct (Bunlungsup et al., 2016; Matsudaira et al., 2018; Osada et al., 2021; Phadphon et al., 2022). Thus, it was hypothesized (as mentioned in Chapter III) that genetic predisposition might play a critical role in the emergence and development of the stone-tool use behaviors in wild long-tailed macaques (Gumert et al., 2019; Reeves et al., 2023).

In general, the emergence and development of stone-tool use behaviors could also be affected by ecological and cultural factors (Gumert et al., 2019). Environmental conditions may be a crucial factor in driving the behavioral divergence observed between species or subspecies. Based on the distribution range, *Mfa*

subspecies inhabited coastal and estuary habitats to a greater extent than the overall *Mff* populations. Marine resources such as oysters, snails, mollusks, and invertebrate animals were abundant in a coastal environment. Thus, the ecological conditions appeared to be conducive to the selection of stone-tool use behavior through natural selection. A comparable occurrence was also observed in capuchin monkeys residing on islands where they developed the capacity to exploit marine resources (Barrett et al., 2018), which might be due to achieving a sustainable number of nutritional requirements in these habitats. The development of stone-tool use behavior could include exposure to learning opportunities (culture), such as role models among social partners as well as the way their activities shape the environment (Gumert et al., 2019; Reeves et al., 2023; Tan et al., 2018).

During the COVID-19 pandemic in July 2022, a survey of the *Mff* population living in the Gulf of Thailand, namely Koh Ped (KPE), was conducted. The island was in close proximity to Pattaya, a city popular for tourism in Thailand. Several hundred macaques have reportedly lived on KPE for at least 60 years. Due to the scenic view of KPE, the tourists arrive on the island daily by boats and yachts. They daily provided the monkeys with diverse foods such as mangoes, cucumbers, nuts, and watermelons. As a result, the monkeys were well habituated to the presence of humans. Although the KPE was visited by Thai researchers several times during these past 10 years to observe the population (Suchinda Malaivijitnond, personal communication), they had never seen stone-tool behaviors in these monkeys.

During my first survey of the KPE population after the COVID-19 travel restrictions were lifted, conducted between 12 – 16 July 2022, it was observed for the first time that two adult males performed stone-tool use behavior to open oysters. The

seashore of the island is covered mainly with rock oysters (*Saccostrea forskali*) (Krabuansang et al., 2020) attached to the rock outcrop. Stone pebbles suitable for stone-tool use were widely abundant along the shore. The monkeys on KPE were assumed to have started using stone-tools to access encased foods such as oysters during the COVID-19 pandemic; however, the exact time it first emerged remains unknown. Here the genetic background of the KPE macaque population, *Mff* subspecies, was confirmed using mtDNA and *SRY* gene sequence, and the morphological characters were identified. The preliminary assessments of the demographic distribution (age and sex) of the new tool behavior on KPE macaques were presented.

Methods

Ethical statement

The permit for sample collection was approved by the Department of National Parks, Wildlife, and Plant Conservation of Thailand. The IACUC of the National Primate Research Center of Thailand-Chulalongkorn University approved the experimental protocols of this study (Protocol Review no. 2075007). The research adhered to the American Society of Primatologists Principles for the Ethical Treatment of NHPs.

Study sites and monkey observation

KPE (GPS: 12°45′N, 100°50′E), also known as Koh Klet Kaew or Monkey Island, is situated on the eastern coast of Thailand, in the Gulf of Thailand (Fig. 4.1). The small island has a shoreline of 2.78 km, covering an area of 0.24 km^2. The island is located only about 600 m away from the mainland, close to a tourist hotspot, Pattaya. Several hundreds of long-tailed macaques have lived on the KPE, which is under the authority of the Royal Thai Navy. The animals highly and regularly interacted with humans, such as climbing on the human's shoulder or head and being provided human foods directly by hand.

The KPE was revisited on the 12 – 15 March 2023, 0800 – 1800 h, in total 1,030 min, and pictures and video footage using a Nikon COOLPIX W300 camera (Nikon, Japan) were taken. When the animals were not in sight, walking along the seashore where the oyster beds are located was done. The evidence of stone-tool use, for example, a stone placed on top of a larger rock with pieces of oyster shell and evidence of use-damage on a hammerstone or anvil was searched. Once the monkeys

were located, they were followed, and a minimum distance of at least 3 m was kept (Kumpai et al., 2022). Each stone-tool use behavior displayed by any individual was filmed, resulting in a total of 31 min of video footage. Apart from the rocky seashore, monkeys were also followed on the beach and on the fringe of the forest to observe the consumption of other foods including plants, which were collected and identified afterward. If more than two monkeys appeared on the seashore, the scan sampling method was used, and a focal animal was selected for foraging behavior.

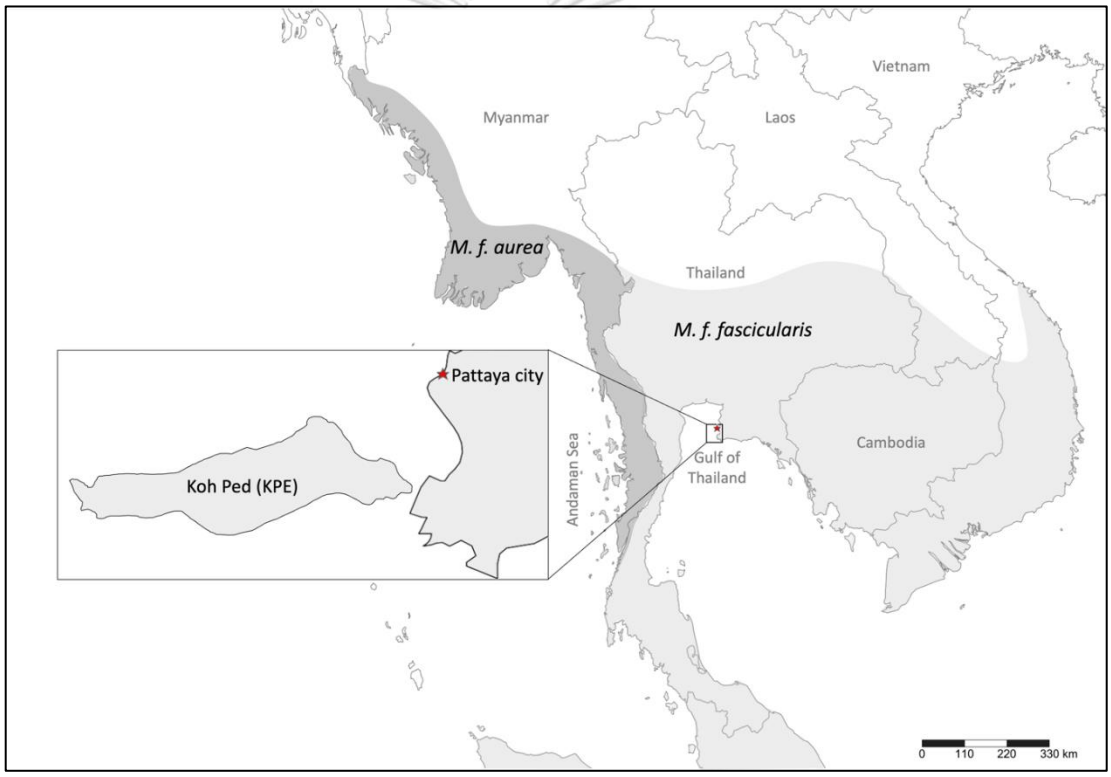

Figure 4. 1. The geographic distribution range of *Mfa* (dark grey) and *Mff* (light grey) and the location of Koh Ped and Pattaya city in Thailand.

Morphospecies identification and specimen collections

Based on the distribution range (Fooden, 1995), these macaques were identified as *Mff.* The subspecies of these *M. fascicularis* were confirmed by morphological characteristics, mainly their cheek hair pattern, vertex of head crest and pelage color (Fooden, 1995; Bunlungsup et al., 2016; Phadphone et al., 2022). *Mff* has a brighter pelage color; the lateral facial crest hairs sweep upward from near the angle of the jaw to the lateral margin of the crown. The hairs of the temporal region are anteriorly directed, so-called transzygomatic pattern. Head crests are either present or absent. Unlike *Mff, Mfa* has a darker dorsal pelage color and no head crest. Furthermore, they exhibit an infrazygomatic pattern of the lateral facial crest hairs: the crest occurs near but inferior to the mandibular region and terminated superiorly in a whorl shape on the cheek, and hairs of the temporal region are posteriorly directed from an eye to an ear (Fooden, 1995; Bunglungsup et al., 2016).

Thirty-one fecal samples were randomly and non-invasively collected from defecated excretions. The feces were swabbed using a cotton swab and stirred in 1.5 mL lysis solution (Hayaishi & Kawamoto, 2006) (0.5% (w/v) SDS, 100 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0 and 10 mM NaCl). The swab was done at the surface of the feces for GI cell collection. To maximize several GI cells harvested, these steps were repeated 3 times per sample before storing the solution at room temperature until DNA extraction.

PCR amplification and sequencing of partial mtDNA and *SRY* gene

The gDNA was extracted from the fecal samples using the QIAamp DNA/Fast DNA Stool Mini kit (QIAGEN Inc., Hilden, Germany) following the manufacturer's

protocol. The mtDNA was amplified using HVS-F (5′-CCGCCCACTCAGCCAATTCCTGTTCT-3′) and HVS-R (5′-CCCGTGATCCATCGAGATGTCTT-3′) primers (Bunlungsup et al., 2016), of which the product size was 835 bp covering the hypervariable segment I (HVSI) of the D-loop region, tRNA proline, tRNA threonine, and cytochrome b. The amplification was carried out at 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and ended up with 72°C for 5 min for final elongation.

The partial *SRY* gene was amplified using *SRY*-FN (5′-TCGCAGCCTCCTTGTTTTTGA-3′) and *SRY*-RN (5′-TCATGGGTCGCTTCACTTTATCC-3′) primers (Phadphon, 2022). The amplification was carried out at 94°C for 1 min, 40 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. Within 216 bp of *SRY* sequences, two polymorphic sites (nos. 42 and 132 of *SRY* Refseq of *M. mulatta* (NM 001032836.1)) were acquired and used to identify *Mfa* (T&C), *Mff* (A&T) and *M. mulatta* (T&T), respectively. PCR mixtures of mtDNA and *SRY* amplification contained 0.5 U ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.3 mM of each primer and 50–100 ng DNA template in the manufacturer's buffer.

The PCR products were run on 2% (w/v) SYBR Safe stained agarose gel-TAE electrophoresis and visualized under the Nucleic acid Bioimaging Instrument (NαBI) blue illuminator (Neo Science Co. Ltd., South Korea). The PCR amplicons were cleaned up using ExoSAP-IT™ (Thermo Fisher Scientific, USA) and purified using BigDye XTerminator™ (SAM solution, BigDye XTerminator™bead solution)

following the manufacturer's protocol before submitting to Macrogen, Inc. (South Korea) for sequencing with the same primer sets.

Phylogenetic tree analysis of mtDNA sequences

The mtDNA sequences were trimmed and aligned using BioEdit 7.2 (Hall, 1999), and the phylogenetic trees were constructed with the Maximum Likelihood (ML) and Bayesian Inference methods. Thirty-one sequences of mtDNA at 573-bp size were analyzed. The GenBank-retrieved Chinese *M. mulatta* sequence (LC093173) and *M. sylvanus* sequence (NC002764) were included in the analysis as outgroups, while the other sequences were retrieved from Bunlungsup et al. (2016) and (2017). The best substitution model was selected based on Bayesian information criterion (BIC) using MEGA X (Kumar et al., 2018). The ML tree was constructed under HKY+G model with 1,000 bootstraps in MEGA X (Kumar et al., 2018). The Bayesian tree was constructed under the same model by using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The analysis was run for 1,000,000 generations, and parameters were sampled every 500 generations. The convergence of the MCMC runs was checked in Tracer 1.5 (Rambaut & Drummond, 2009) with the trace plot and over 200 effective sample size (ESS) values for all parameters. The first 25% of data were discarded as burn-in, the remaining data were combined, and a 50% majority-rule consensus tree with posterior probability on each branch was summarized. The tree was visualized in FigTree 1.3.1. (Rambaut, 2010).

Results

Phenotype and genotype identification

By morphospecies identification, all monkeys on KPE had transzygomatic cheek-hair patterns and a head crest (Figure 4.2A and B), as seen in *Mff* (Fooden, 1995; Bunlungsup et al., 2016). Their pelage color was light, as seen in Indochinese *Mff* (Figure 4.2C; Hamada et al., 2008). Thus, the KPE monkeys were morphologically identified as Indochinese *Mff*.

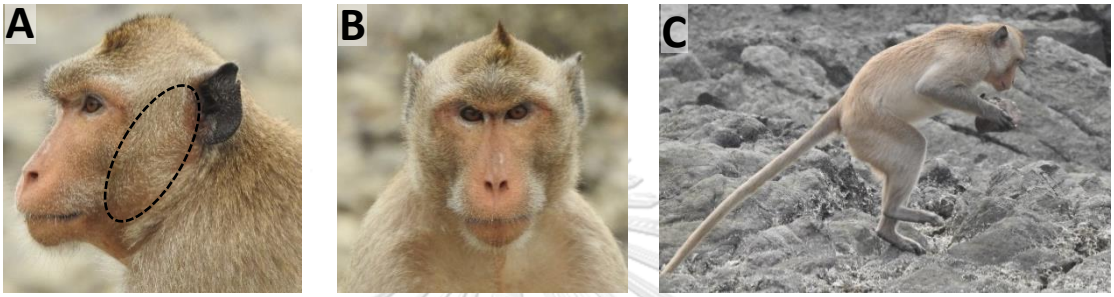

Figure 4. 2. Adult male with transzygomatic cheek hair pattern (dashed circle) **A)**, head crest **B**) and light pelage color **C**).

All 573-bp mtDNA sequences analyzed showed 100% homology, thus only three KPE mtDNA sequences were included in the phylogenetic analysis. The ML and Bayesian phylogenetic trees of mtDNA showed a similar topology; therefore, only the Bayesian tree was used in this study (Figure 4.3). The tree indicated the divergence of the *Mfa* clade from the *M. mulatta*/*Mff* clade before the divergence of the *M. mulatta* and *Mff* clades. As for the *Mff* clade, there were two subclades separated by the Isthmus of Kra (10° 15’ N, 99° 30' E); the northern Indochinese and the southern Sundaic subclade. KPE monkeys belonged to the Indochinese *Mff* subclade.

Regarding the analysis of the 216 bp of the *SRY* gene, 12 of 31 samples could be amplified and sequenced. Two variable sites (no’s. 42 and 132 of *SRY* Refseq of *M. mulatta*) in all 12 animals were T and T, respectively, which indicated the *M. mulatta*

haplotype, and this was named as *M. mulatta*/Indochinese *Mff* clade in Bunlungsup et al. (2016, 2017).

Taken together for the mtDNA and *SRY* gene analysis, KPE monkeys were genetically identified as *Mff* subspecies of the Indochinese form.

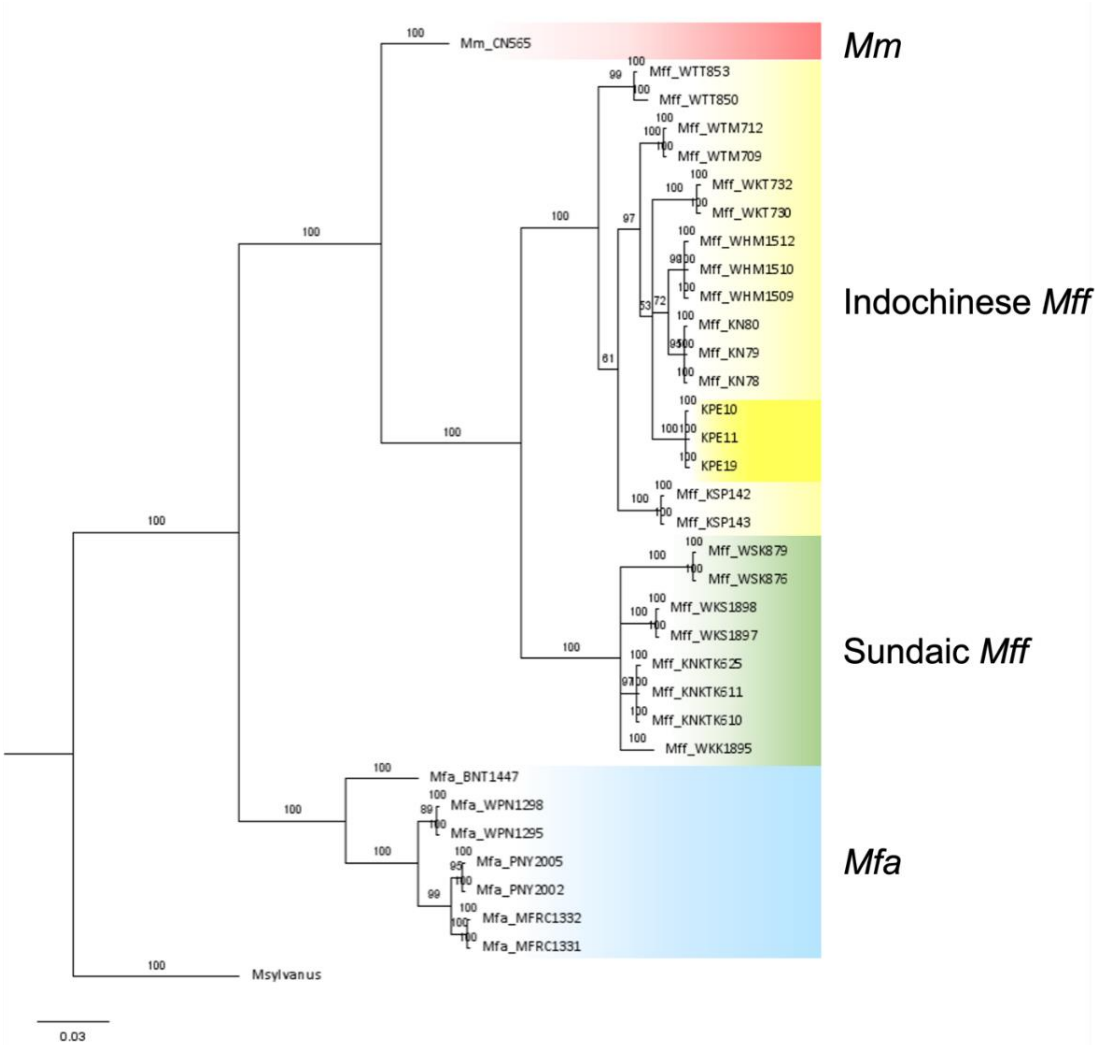

Figure 4. 3. Bayesian phylogenetic tree based on 573 bp of the mtDNA gene. The tree's three-letter codes correspond to those in Bunlungsup et al. (2017) (Bunlungsup et al., 2017). Dark yellow color indicates the KPE samples that were analyzed in this study. The numbers on each branch refer to the posterior probability/bootstrap values.

Stone-tool use behavior

During the (re)visit in March 2023, when the restrictions of COVID-19 were lifted, and tourists occasionally visited KPE island, the evidence of stone-tool use, i.e., pieces of oyster shells and stones with evidence of percussive use-damage on the oyster bed or rock anvils was searched. The key personnel of the Royal Thai Navy and the boat drivers were also interviewed. They reported that they had never seen monkeys using stones to crack open foods. Monkeys roamed freely and were observed to forage for foods categorized as natural or human-provisioned foods. Natural foods included invertebrate animals, i.e., crabs, rock oysters (*Saccostrea forskali*), venus clam, and commercial bivalves (*Gafrarium tumidum*), and plants, i.e., catappa nut (*Terminalia catappa*), mangrove pods (*Rhizophora sp.*), young buds of Bantigue (*Pemphis acidula*) and young fruit of *Chaetocarpus castanocarpus*. Human-provisioned foods included sea fish, banana, cucumber, carrot, purple cabbage, pineapple, watermelon, spring onion and ripe mango.

Seventeen animals were identified using percussive stones to crack open the rock oysters attached to the rock anvil (Figure 4.4 and Table 4.1). All of them were subadults and adults, and 15 out of 17 were males. One adult male monkey was seen to perform the behavior twice, on 12 and 13 March 2023, and this individual was counted as one animal. Compared to other stone-tools using macaque populations (Gumert et al., 2009; 2011; Tan et al., 2018), these animals did not manipulate the stone well; mostly, they held the large stones (weighing >1 kg) with two hands while they were sitting, raised the hands up not higher than their shoulder, and threw stone onto the oyster bed. This stone manipulation was named in this study as “pound-hammering-like”. Once the shell cracked, monkeys used their hand(s) or teeth to open

the oysters (or gnaw manipulation in Bandini and Tennie, 2018) to consume the meat. Some monkeys threw the stone more randomly, not directly targeting the oysters. Mostly adult males performed the behavior while they were foraging in solitary.

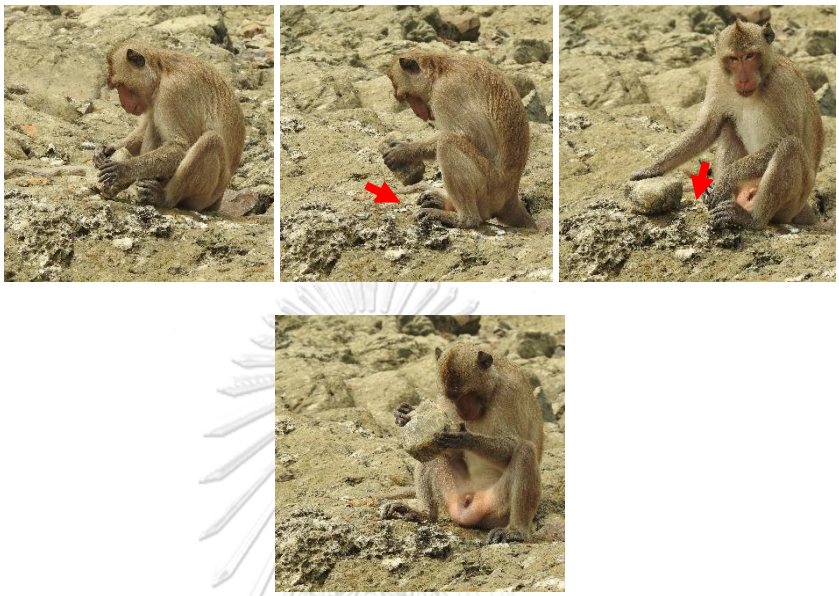

Figure 4. 4. A series of rock oysters cracking in an adult male. The rock oysters (red arrow) were attached to the rock anvil, and an animal used percussive stone-tool to open the oysters.

Table 4. 1. Sex and age-class of stone-tool used macaques observed on KPE Island.

Sex	**Male**		**Female**	
Age-class	**Adult**	**Sub adult**	**Adult**	**Sub adult**
12 March 2023	2	-	-	-
13 March 2023	5 (+1*)	1	1	-
14 March 2023	7	-	1	-
All	14 (+1*)	1	2	-

*This adult male was observed to perform stone-tool use behavior twice, on 12 and 13 March 2023. He was not repeatedly counted on 13 March 2023.

Discussion

This is the first report of the stone-tool use in *Mff*, which was therefore only the sixth wild NHP species observed to perform this behavior following West African chimpanzees (*Pan troglodytes* verus; Sugiyama and Koman, 1979), bearded capuchins (*Sapajus libidinosus*; Fragaszy et al., 2004), *Mfa* (Malaivijitnond et al., 2007), yellow-breasted capuchins (*S. xanthosternos*; Canale et al., 2009), and white-faced capuchins (*Cebus capucinus imitator*, Barrett et al., 2018). They were identified as *Mff* subspecies based on distribution, morphological and genetic (mtDNA and *SRY* gene) characteristics. Following Bunlungsup et al. (2017) revealing the past introgression of *M. mulatta* males to Indochinese *Mff* population, where the introgression was terminated at the vicinity of Isthmus of Kra, and the Indochinese *Mff* were harbored the *M. mulatta* Y-chromosome gene, thus in this study the two *SRY* gene SNPs (T and T) analyzed confirmed that the KPE monkeys belonged to the Indochinese form of *Mff* subspecies. In the past, only *Mfa* (Malaivijitnond et al., 2007) and hybrids between *Mff* x *Mfa* (Luncz et al., 2017; Tan et al., 2018) were reported to display stone-tool use behaviors as stated in Chapter I, II and III. Even though it was a training program, captive *Mff* were unable to develop the behavior (Bandini and Tennie, 2018). It was therefore concluded that the genetic predispositions of *Mfa* played a crucial role in the emergence and development of this behavior in long-tailed macaques (Bandini and Tennie, 2018; Gumert et al., 2019). Thus, the discovery of "pound-hammering-like" behavior in KPE *Mff* in this Chapter IV opposed this conclusion. That is, the genetic predispositions of *Mfa* are not an absolute prerequisite for the emergence of stone-tool use behavior in long-tailed macaques. Although the KPE macaques were identified as *Mff*, the genetic

contributions of *Mfa* to this population could not be ruled out. Using mtDNA and *SRY* genes has some disadvantages as it cannot predict the level of genetic admixture of *Mfa* in *Mff* (Bunlungsup et al., 2016). For example, two populations of *Mff* living in the northern region of Thailand (GPS: 15°56′N and 13°02′N; Phadphon, 2022) were identified carrying 11% and 14% of *Mfa* genetic ancestry analyzed by 868-autosomal SNPs. Thus, the level of genetic admixture of *Mfa* ancestry in the KPE population should be assessed by autosomal SNP markers in the near future.

The composition of the animal's habitat, such as the availability of suitable stone materials; potential hammerstones and anvils, as well as the presence of encased foods (i.e., oysters in KPE Island), enabled the emergence of the behavior. Based on the discovery of stone-tool use behavior in the KPE-*Mff* during the COVID-19 episode, it indicated that even though optimal environmental conditions for tool use have existed for several decades, without the pressure of food scarcity, this specific behavior would likely not have developed (Bandini and Tennie, 2018). Thus, food scarcity should be the main driving factor for the emergence of stone-tool use behavior in long-tailed macaques. This finding supports "the necessity hypothesis" proposed by Fox et al. (1999) for tool use among orangutan populations where tool use is maintained by sustenance needs during resource scarcity (Fox et al., 1999). For KPE-*Mff*, the human-provided foods are the most efficient option for sufficient caloric intake. Similarly, food scarcity and starvation risk were also proposed as key factors in the emergence of nut-cracking behavior in yellow-breasted capuchins living in dry forests and thorn scrub in the Caatinga biome, Brazil (Canale et al., 2009; Moura & Lee, 2004). Similarly, chimpanzees used stone-tools to exploit *Coula* nuts more frequently in the dry season, during which their main food sources were scarce

(Boesch & Boesch, 1984). As the oyster-cracking behavior of this KPE population was discovered during the time of COVID-19, the food scarcity over three years should pressure the animals to adapt to their marine environment. Thus, this sheds light on the positive magnitude of COVID-19 in wildlife.

Generally, it might not be common for NHPs to expose marine food sources. *Mff*, however, are familiar with aquatic ecosystems; they are good swimmers (Fooden, 1995). Additionally, KPE-*Mff* were customarily fed by tourists, focusing their attention on the forest to the seashore. Through increased exposure to the shore, the animals could have learned about marine food sources. The observed stone manipulation pattern was named "pound-hammering-like" because the monkeys did not handle the stone throughout the usual cracking processes. They dealt with the stone with two hands, raised their hands to their chests and threw the stone into the oyster beds. Object (tree-branch) throwing was previously observed in a population of wild chimpanzees intending to attack the unfamiliar person approaching (Sugiyama and Koman, 1979). Most chimpanzees that displayed this behavior were also adult males, similar to the observed stone-throwing in KPE-*Mff*. It might be that, in some situations, an adult male *Mff* threw a stone into an oyster bed, accidentally cracked opened the oyster shells, saw the broken shell, and accessed oyster meat afterward.

Based on the actions of stone-throwing described in the literature, it was proposed that the "pound-hammering-like" behavior in KPE population originated from uncoordinated stone-throwing (the first step before stone-tool use) and modified it over time to percussive stone-tool use. Stone throwing was also described in West African chimpanzee (*Pan troglodytes verus*) (Kühl et al., 2016), Japanese macaque (*M. fuscata*) (Leca et al., 2008), chacma baboon (*Papio ursinus*) (Hamilton et al.,

1975) and bearded capuchin monkeys (Falótico & Ottoni, 2013). Stone throwing has been reported most frequently in NHP species that used tools in other contexts, i.e., capuchin monkeys and chimpanzees. Stone-throwing in Japanese macaque has been listed as one of the numerous behavioral patterns of the stone-handling repertoire of this species (Leca et al., 2007; Leca et al., 2008). Compared to Japanese macaques, the closest living NHP species to *Mff*, stone's throwing styles in Japanese macaques were generally "underarm throwing", which is different from what was observed in KPE-*Mff*. The stone-throwing of Japanese macaques was performed from a tripedal posture (one-handed sequential-movement operation) and often accompanied by repeated jumps (Leca et al., 2008), while the stone-handling in KPE-*Mff* was performed with two hands while sitting, which looked similar to "pound-hammering" which is more advanced than the stone-throwing. The directions of stone material thrown by Japanese macaques were backward, upward, sideways, or forward (Leca et al., 2008), while KPE-*Mff* threw stones vertically and directed downwards. Throwing of stones in KPE-*Mff* at the end of pound-hammering-like processes might be a result of the stone being very heavy (>1 kg) compared to their body weight (adult males, ranging 5-8 kg; Hamada et al., 2008) which was far beyond their capacity to hold it for a long while they were sitting. However, throwing causes a loss of accuracy. From a functional viewpoint, the stone-throwing observed in Japanese macaques was regarded as a non-instrumental manipulation of stones (Leca et al., 2008), while the pound-hammering-like in KPE-*Mff* was a targeted (oyster) foraging activity. Because this stone-tool use behavior only recently emerged in the KPE population, it is interesting to investigate further if it evolved from non-functional stone-throwing into functional stone-tool use. It will be important to further observe the macaques of KPE

to investigate if the animals will modify the stone manipulation style and select smaller and lighter stones for oyster cracking, as seen in the pureblood *Mfa* living at Piak Nam Yai island, southwestern Thailand (Gumert et al., 2009; 2011). Regarding the heavy stone use, it might also explain why the oyster-cracking behavior was observed only in KPE-*Mff* adults/subadults and 88% were males.

As mentioned above, the *Mfa* genetic ancestry is not a critical factor for stone-tool use emergence in long-tailed macaques, however, the genetics might play a role on stone-tool use proficiency (or stone-tool use development) in this species. Comparing to *Mfa* and *Mfa* x *Mff* hybrids (Gumert et al., 2011; Lydia V Luncz et al., 2017; Tan et al., 2015; Tan et al., 2018), the "pound-hammering like" behavior in KPE-*Mff* was less proficient on targeting the objects (oyster foods in this case). Therefore, genetics might contribute to the stone-tool manipulation skill in long-tailed macaques. A long-term study of this population should be conducted and observed if the pound-hammering-like behavior will remain the same or if it will develop to a more advanced level, such as axe hammering, as seen in *Mfa* and *Mfa* x *Mff* hybrids.

How the behavior spread throughout the KPE group is still unknown. However, since many KPE males were observed to perform this behavior while were solitary, it might have been difficult for other animals to observe and learn. Tan et al. (2018) reported that young long-tailed macaques preferred to learn to use stone-tools from closer, older, and better tool users. Previously, Bandini and Tennie (2018) experimentally tested captive *Mff* by motivating them with provided ecological materials necessary for pound-hammering, i.e., hammering stones, shelled nuts, and stone anvils. However, the captive *Mff* did not perform the pound-hammering behavior, even not after repeated demonstrations. The researchers concluded that the

Mff could not learn pound-hammering and that the levels of individual learning abilities and motivation to attend to socially mediated information of *Mff* differed from those of *Mfa*. However, the present study shows that wild *Mff* can develop stone pound-hammering-like behavior if the motivation is strong enough to encourage the acquisition. In this case, at least two years (2020-2022) of exposure to severe food scarcity due to the COVID-19 pandemic is the cause. Besides, it suggests that the emergence of stone-tool behavior is not related to a critical period of learning, at around 3 years of age, as seen in wild juvenile *Mfa* x *Mff* hybrids (Tan et al., 2018) because, in the KPE population, only subadults/adults were observed the behavior.

As seen in the stone-tool use in *Mfa* at Piak Nam Yai island, southwestern Thailand (Gumert et al., 2011) and *Mfa* x *Mff* hybrids at Koram Island, southern Thailand (Luncz et al., 2017; Tan et al., 2018), and the stone-throwing in Japanese macaques, stone-manipulating behaviors were suggested to transmit from one generation to the next generation via maternal kinship and social proximity (Leca et al., 2008). “Pound-hammering-like” behavior observed in KPE females was rare (only 2 out of 17 stone-tool users). The newly emerged tool behavior might disappear again quickly after tourism has returned to the island and people start provisioning the animals again. It might be a tragedy for science because many research questions await investigation. For example, how many populations on the islands can use stone-tools? How does the new stone-tool behavior spread amongst group members and between groups? What is the feeding range between stone-tool users and non-stone-tool users? Do they use stones to crack other food types and what is the manipulation style? What are the changes in size, shape and weight of selected stone-tools? What does the efficiency of stone-tool use in KPE-*Mff* compared to the pureblood *Mfa* and

the *Mff* x *Mfa* hybrids? (Gumert et al., 2011; Gumert et al., 2009; Gumert et al., 2019), and if no human disturbance occurs, whether this behavior can develop to the more proficient level as seen in wild *Mfa* monkeys?

CHAPTER V

ASSOCIATION OF PLASMA TRYPTOPHAN AND 5-HT LEVELS IN *Macaca fascicularis fascicularis* AND *M. f. aurea* AND STONE-TOOL USE BEHAVIORS

Introduction

Macaca fascicularis is a species of Old-World monkey that inhabits tropical Southeast Asia (Fooden, 1995). *Mfa*, along with chimpanzees and capuchins, held notable significance among NHP species due to their distinctive behavior of using percussive stone-tools for foraging purposes (Boesch & Boesch, 1981; Canale et al., 2009; Fragaszy et al., 2004; Malaivijitnond et al., 2007). *Mfa* originated in Myanmar, extended its distribution southeastward across the Mergui Archipelago to southwestern Thailand, and lived in proximity with *Mff* (Bunlungsup et al., 2016; Fooden, 1995). The distribution range of *Mfa* covered most of the coastal and estuary habitats, leading to the dietary sources that need the stone-tools. In Chapter IV, the first *Mff* population (KPE monkeys) living on the island was observed using stone-tools to crack open oysters during the COVID-19 food scarcity, and in Chapter III, *Mff* and *Mfa* lived in different habitat types consumed variations in food items and had different compositions of gut microbiota. Subsequently, the different food types deriving from different habitat types, presence/absence of stone-tool use behaviors and different genetic predisposition of animals, might influence the gut-brain axis (Forsythe et al., 2010; Grenham et al., 2011) and potentially affect learning and cognitive processes through the production of diverse metabolites derived from their food metabolism (Cryan & Dinan, 2012; Sherwin et al., 2016).

In the last decades, an extensive research effort has been dedicated to unraveling the evolutionary processes behind the unique characteristics of the hominin brain, marked by a substantial increase in size and complexity compared to other primates (Schoenemann, 2006). The evolution of the human's brain experienced an increase in size and complexity, starting from *H. habilis* with the emergence of Oldowan stone-tool manufacturing, continuing with *H. erectus* and the use of Acheulean stone-tools, and reaching its peak in *H. neanderthalensis* and *H. sapiens sapiens* with the development of Mousterian stone-tools. During this 2.5-million-year hominin evolution, the brain size was triple which was distinguish humans from other NHPs that maintained relatively stable brain sizes (Bretas et al., 2019). This leads to the hypothesis that the stone-tool use should ascribe variances in brain development via the gut-brain axis. The gut-brain axis is bidirectional communication between the CNS and ENS (Cryan & Dinan, 2012; Forsythe et al., 2010), and multiple pathways, including metabolic pathway, i.e., Trp metabolism (Agus et al., 2018).

Trp is an essential amino acid, which serves as a precursor for 5-HT (Rapport et al., 1948). 5-HT is a crucial neurotransmitter involved in modulating central neurotransmissions maintaining mood as well as cognition (Cryan & Leonard, 2000), and enteric physiological functions regulating GI secretion and gut motility (Costedio et al., 2007; McLean et al., 2007), thereby exerting its regulatory effects across the gut-brain axis. The synthesis of 5-HT in the brain relies on the presence of its precursor Trp, and Trp levels in the CNS are influenced by the availability of Trp obtained from the diet (Berger et al., 2009; Rapport et al., 1948). When Trp level was depleted through restricted diets, both mice and healthy human volunteers experienced lower Trp levels in their blood, which consequently led to reduced 5-HT

levels in the CNS (Browne et al., 2012; Moore et al., 2000). The gut microbiota found in the digestive system appears to influence the availability and metabolism of Trp, and indirectly affects 5-HT levels in the brain (Desbonnet et al., 2008). Therefore, to gain a better understanding of a plausible involvement of Trp and 5-HT in gut-brain axis, especially the brain development, in association with food acquisition via stone-tool use behaviors, plasma Trp and 5-HT levels in two *M. fascicularis* populations, the non-stone-tool users (*Mff*) and stone-tool (*Mfa*) users, living in the same habitat type (mangrove forest) were compared.

Methods

Ethical note

The study conducted in Thailand involving two populations of free-ranging long-tailed macaques obtained necessary research permits and sample collection approvals from the Department of National Parks, Wildlife, and Plant Conservation of Thailand. The IACUC of the NPRCT-CU approved the study's experimental protocols (Protocol Review no. 2075007). The research followed ethical guidelines outlined by the ASP for the ethical treatment of NHPs. All methods employed in the study adhered to relevant guidelines and regulations.

Study sites and plasma sample collection

The study sites were Mangrove Forest Research Center (MFRC), Ranong province (GLP: 9°52′, 98°36′) where the *Mfa* stone-tool users lived, and Bang Ta Boon (BTB), Phetchaburi province (GLP: 13°15′, 99°56′) where the *Mff* non-stone-tool users lived. Both study sites were mangrove forest habitats. Sixteen MFRC-*Mff*

and 45 BTB-*Mfa* were temporarily captured (see below) and collected blood samples. The subspecies were identified based on their morphological characteristics, pelage color, cheek hair pattern and geographic distribution (Fooden, 1995; Bunlungsup et al., 2016; Phadphon et al., 2022).

Animals were captured using iron mesh traps (Fig. 5.1 a-c) and were anesthetized by intramuscular injection with 2–5 mg/kg of tiletamine/zolazepam (Virbac, France) mixed with 20–50 ug/kg of (dex) medetomidine hydrochloride (Zoetis, USA) (Fig. 5.1 d) (Malaivijitnond & Hamada, 2008). The animal's body weight was measured and recorded (Fig. 5.1 e). The captured animals were separated into three-age classes based on their estimated ages from the dental eruption: adult (> 6 years), sub adult (3–6 years), and juvenile (< 3 years) (Meesawat et al., 2023). Thus, MFRC-*Mff* comprised 10 adults, 4 subadults and 2 juveniles, and BTB-*Mfa* consisted of 20 adults, 15 subadults and 10 juveniles, respectively.

Blood samples (3 mL/kg BW, but not more than 10 mL/individual) were collected by femoral venipuncture and kept in EDTA tubes (Fig. 5.1 f). The plasma samples were separated by centrifugation at 2000 x*g* for 15 min, transferred to the laboratory, and kept at -20 °C until analysis. After collecting specimens, an intramuscular injection of atipamezole hydrochloride (Zoetis, USA) at the same volume as the (dex) medetomidine hydrochloride anesthetic dose was administered. The macaques were released back to their natural habitats upon fully recovering from anesthesia.

Figure 5. 1. The procedure for capturing the free-ranging monkeys in their natural habitats. **a**) The utilization of iron mesh traps (1.7 x 3-15 x 1.7 meters), where the set-up consisted of a 3-chamber interconnected design. **b**) The deployment of automatic box traps. **c**) Transferring the captured monkeys to transferring cages. **d**) Administering anesthesia to captured monkeys. **e**) Measuring the body mass of the monkeys and attaching identification tags. **f**) Blood sample collection by femoral venipuncture.

Plasma sample preparation

Plasma samples (150 μL) were deproteinized by adding 10 μL of 35% perchloric acid. The acidified plasma was immediately vortexed for 1 min, incubated at room temperature for 10 min, and centrifuged at 15,000 x*g* at 4 °C for 10 min. Later, 50 μL of the supernatant was collected and injected into the HPLC system for analysis.

Preparation of Trp and 5-HT standards

Standard Trp and 5-HT were purchased from Sigma (St. Louis, MO, USA). Chromatographic grade acetonitrile was purchased from Burdick and Jackson (Muskegon, MI, USA). All other chemicals were of analytical grade, and solvents were of chromatographic purity. Chromatographic grade water was prepared using a MilliQ^{TM} system (Millipore, MA, USA).

The standard stock solutions of Trp and 5-HT were prepared in 10 mmol/L sodium acetate–acetic acid (pH 4.5) at 20.0 mmol/L and 5.0 mmol/L, respectively, and stored at −20 °C. The standard working solutions were prepared by diluting each stock solution with 10 mmol/L sodium acetate – acetic acid to produce the concentrations range of 25–400 μmol/ L for Trp and 5–1000 μmol/L for 5-HT.

HPLC analysis

An Agilent 1260 Infinity II (Santa Clara, CA, USA) HPLC system was used in this study which was equipped with a quaternary pump (VL G7111A), vial sampler (G7129A), and diode array detector (G7115A). An Agilent Eclipse XDB-C18 (4.6 μm x 250mm, 5 μm) column was used for analysis at 25 °C. The chromatographic

separation was carried out using a mobile phase consisting of 10 mmol/L sodium acetate – acetic acid (pH 4.5) and acetonitrile (94:6, v/v) at a flow rate of 0.6 mL/min following a previous method (Zhen et al., 2011). The eluates were monitored by the programmed wavelength detection setting at 220 nm from 0 min to 7.5 min for 5-HT and from 7.5 min to 12.0 min for Trp.

Data analysis

The data was acquired and processed with Agilent Chemstation software. The data were presented as mean ± standard error (Table 5.1), while the box and whisker plot was used to represent the distribution of dataset. The box in the plot represented the interquartile range (IQR), with the bottom of the box indicated the 25th percentile (Q1) and the top of the box represented the 75th percentile (Q3). The whiskers represented the outliers present in the dataset. The data normality test was applied for both BTB-*Mff* and MFRC-*Mfa* populations. Following the normality test, the non-parametric test (Kruskal-Wallis test) was used to analyze the statistical differences of Trp levels between three age-classes of BTB population, while the parametric test (ANOVA) was used for the MFRC population, Comparison between BTB-*Mff* and MFRC-*Mfa* populations was done by independent sample t-test. All the statistical analysis was performed in SPSS (IBM, SPSS Inc. USA) version 28.0 and OriginPro 2023b (OriginLab Corporation, USA) software packages. A significance level of $P < 0.05$ was considered significant.

Results

Chromatogram of a standard mixture of 5-HT and Trp

The 5-HT and Trp could be separated at 6.287 min and 10.667 min, respectively (Fig. 5.2). Linear regressions (r) calibration curve for 5-HT and Trp were 0.99995 and 0.99971, respectively. The generated calibration curve was used for measuring the concentration of 5-HT and Trp in the plasma sample of *Mff* and *Mfa*. Following the current method, the plasma Trp levels in *Mff* and *Mfa* populations were detected, while the plasma 5-HT levels were lower than the detectable limit and undetectable in both *Mff* and *Mfa* populations (Fig. 5.3).

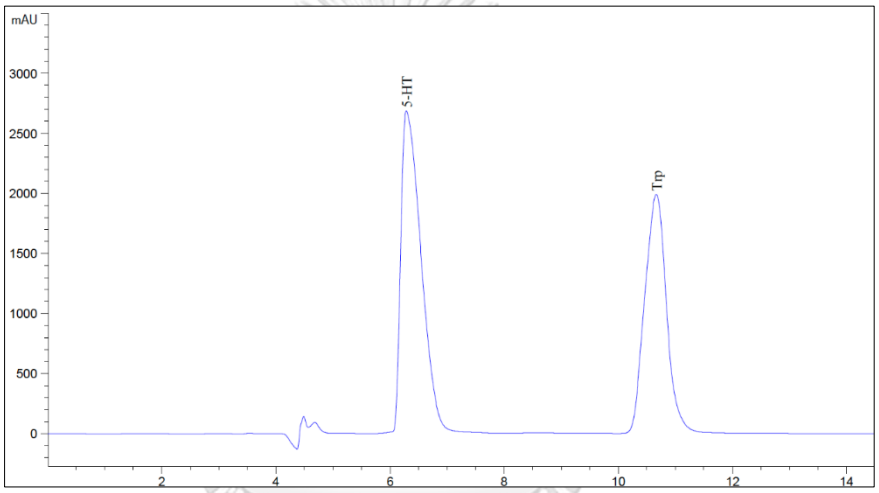

Figure 5. 2. Separation of 5-HT (6.287 min) and Trp (10.667 min) in the standard mixture. Conditions: Eclipse XDB-C18 (4.6 µm x 250mm, 5 µm); mobile phase, 10 mmol/L sodium acetate–acetic acid (pH 4.5) containing 6% (v/v) acetonitrile; flow rate, 0.6 ml/min; injection volume, 50 µl.

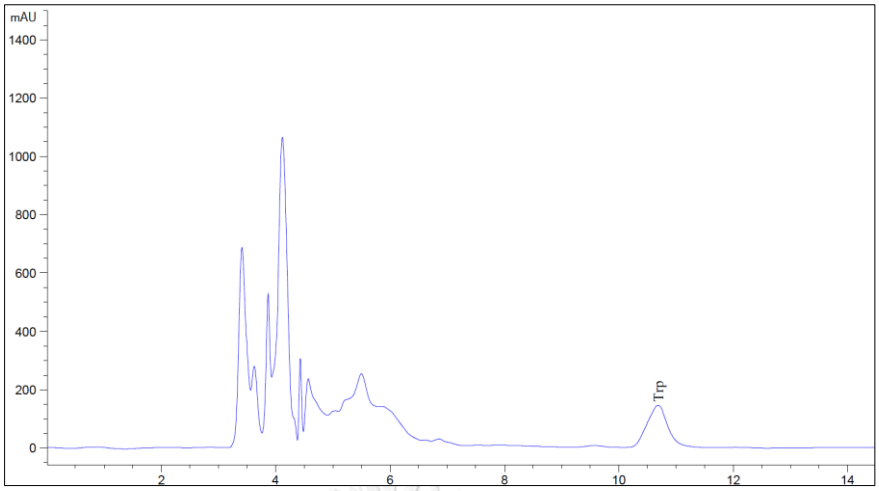

Figure 5. 3. Separation of Trp (10.687 min) in the plasma sample of *Mff*. Conditions were followed in Fig. 5.2.

Plasma Trp levels in *Mff* and *Mfa*

Though plasma Trp level of MFRC-*Mfa* population (28.88 ± 4.3 μmol/L) tended to be higher than the BTB-*Mff* population (23.23 ± 1.4 μmol/L), it was not significant difference (*P = 0.116*) (Fig. 5.4, Table 5.1). When animals were separated into three-age classes (adult, subadult and juvenile) and plasma Trp levels were compared in each population, no significant differences were detected either in BTB-*Mff* (*P = 0.657*) or MFRC-*Mfa* (*P = 0.447*) populations (Fig 5.5 a and b, Table 5.1). When plasma Trp levels were compared between populations in each age-class, however, the MFRC-*Mfa* adults had significantly higher plasma Trp level than the BTB-*Mff* adults (*P = 0.040*), while the levels were non-significantly different in sub adults and juveniles (*P = 0.159* and *P = 0.709*, respectively).

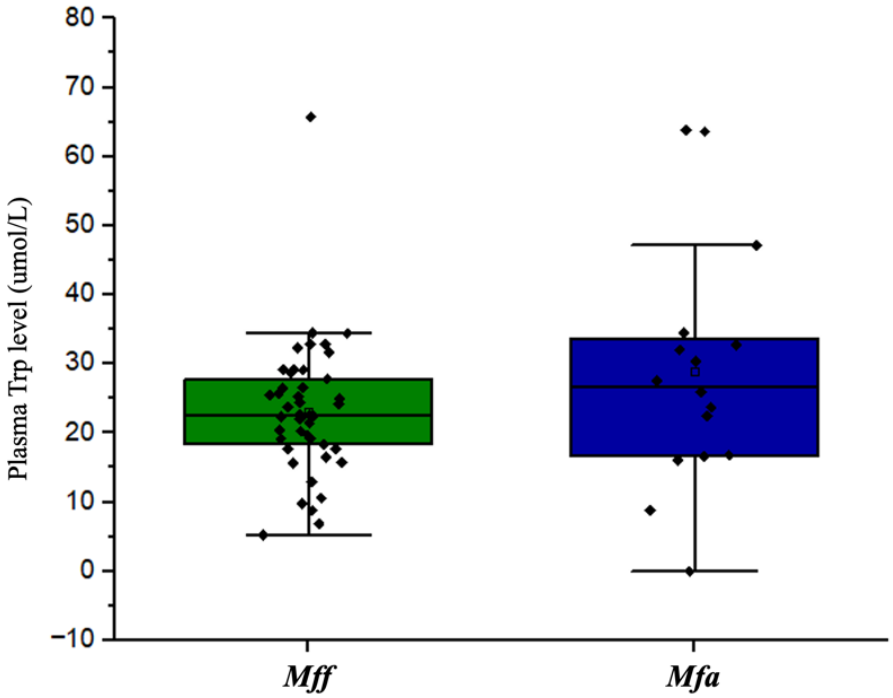

Figure 5. 4. Plasma Trp levels of BTB-*Mff* non-stone-tool user and MFRC-*Mfa* stone-tool user populations. The dataset's distribution is shown in a box and whisker plot, where the box represents the interquartile range (IQR) with the 25th percentile (Q1) at the bottom and the 75th percentile (Q3) at the top. The whiskers indicate the outliers in the dataset.

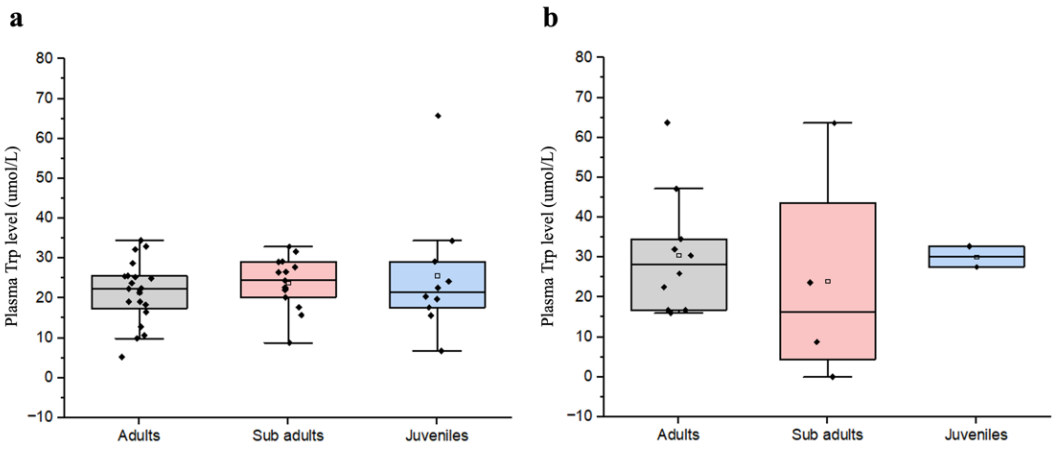

Figure 5. 5. Plasma Trp levels in adults (grey), sub adults (pink), and juveniles (blue) in BTB-*Mff* non-stone-tool user **a**) and MFRC-*Mfa* stone-tool user **b**) populations. The dataset's distribution is shown in a box and whisker plot as described in Figure 5.5.

Table 5. 1. Plasma Trp levels (μmol/L) in three age-class of BTB-*Mff* and MFRC-*Mfa* populations.

No.	BTB-*Mff*			MFRC-*Mfa*		
	Adult (n=20)	**Subadult(n=15)**	**Juvenile (n=10)**	**Adult (n=10)**	**Subadult (n=4)**	**Juvenile (n=2)**
1	22.38	22.68	20.41	16.64	8.78	32.75
2	32.24	8.75	15.61	32.01	23.73	27.55
3	23.78	27.78	19.75	16.05	0.00	
4	24.93	15.72	17.69	30.38	63.61	
5	25.47	20.23	22.57	22.48		
6	22.4	26.46	6.80	34.50		
7	9.83	24.4	29.14	47.14		
8	25.63	29.17	34.40	63.78		
9	19.18	17.71	24.26	25.94		
10	32.91	21.99	65.75	16.76		
11	19.14	22.40				
12	5.27	32.87				
13	10.64	31.70				
14	16.56	29.13				
15	28.71	26.57				
16	21.41					
17	34.46					
18	12.88					
19	18.35					
20	25.29					
Mean±SE	21.5 ± 1.7	23.8 ± 1.6	25.6 ± 5.0	30.5 ± 4.8	16.2 ± 7.4	30.1 ± 2.6

Discussion

This study aimed to compare the levels of 5-HT and Trp in the plasma samples of the non-stone-tool users (*Mff*) and stone-tool (*Mfa*) users, using the HPLC method, to understand an association between the gut and the brain (or gut-brain axis) and food acquisition via the use of stone-tools. The selected two populations of *Mff* and *Mfa* lived in mangrove forests reflecting the similarity in their habitat type, which should provide comparable available food types. Although the overall results revealed no significant differences in plasma Trp levels between *Mff* and *Mfa* populations, the Trp level in MFRC-*Mfa* adults was significantly higher than that in the BTB-*Mff*

adults. This finding aligns with the stone-tool use behaviors observed in the MFRC population, mainly adult monkeys. Since Trp could not be synthesized in monkeys and must be obtained through diet, the availability of Trp-rich foods in the diet can directly influence the plasma Trp levels (Madras et al., 1974; Rapport et al., 1948). Thus, similar habitat types, but differences in food acquisitions via stone-tool use, can provide varying food resources and availability of Trp-rich foods. A significantly higher Trp level in MFRC-adults implies a plausible association between a higher plasma Trp level and the presence of stone-tool use behavior in these animals. Noted, during the field observation, the BTB-*Mff* monkeys were also provided foods by local people, while the MFRC-*Mfa* population depended only on the food sources available in nature. It is interesting to investigate further which food types in the MFRC habitat provide high availability of Trp level and how much the MFRC-adults consume those stone-tool assisted foods compared to other age-class.

Previously, it has been reported in humans that throughout infancy, childhood, and adolescence, the plasma Trp levels exhibited a gradual and steady increase (Lepage et al., 1997). In contrast, another study in humans unveiled that younger individual had higher Trp levels than older individuals (Demling et al., 1996). However, no association between plasma Trp level and age-class (adult, subadult and juvenile) was detected within the MFRC-*Mfa* population, and this might be because the animal numbers were too low, especially subadult (n = 4) and juvenile (n = 2) ages. Thus, more plasma specimens need to be collected, and the underlying mechanisms behind these age-related differences in plasma Trp levels should be investigated further.

Previous study has suggested a coevolutionary relationship between the significant expansions in *Homo's* brain sizes over the past 2.5 million years and the development of stone-tool technology. The utilization of stone-tools by different *Homo* species coincided with a tripled increase in brain size (Bretas et al., 2019). Although the brain sizes of monkeys were not measured in the present study, the skull sizes between BTB-*Mff* and MFRC-*Mfa* adults, based on my observation during field study, were comparable. This suggests that the higher plasma Trp levels observed in MFRC-adults stone-tool users are not associated with brain size expansion, particularly the cerebrum. Noted, Trp serves as a precursor for 5-HT neurotransmitter where its receptors are highly expressed in hippocampus and amygdala functioning in cognition and learning (Meneses, 1999; Berger et al., 2009). Thus, these findings suggest a potential link between stone-tool use, plasma Trp level, and learning and cognitive processes in the MFRC-adults. However, further research is required to establish a definitive causal relationship and gain a deeper understanding of the complex interplay between these factors.

Despite the current study, which was unable to detect 5-HT in the plasma samples of both *Mff* and *Mfa* populations, one potential explanation could be the degradation of 5-HT due to its short lifespan in the plasma (Carling et al., 2002; Thomas & Vane, 1967). Another factor that may have contributed to the undetectable plasma 5-HT levels is the temperature instability during sample storage in the field and transportation to the laboratory (Huang & Kissinger, 1996). Trp can cross the BBB by competing with other large neutral amino acids and be metabolized into 5-HT in the brain (Pardridge, 1979), and the periphery 5-HT could also be taken up and stored by platelets (Ni & Watts, 2006). These processes should help explain a lower,

undetectable 5-HT level in the peripheral circulation of *M. fascicularis* in this study (Berger et al., 2009). Therefore, it is recommended to implement significant precautions to prevent the degradation of 5-HT and select platelet-rich plasma samples for the accurate detection of 5-HT levels in *M. fascicularis* in future studies.

As the production of neural 5-HT relies solely on the presence of Trp (Berger et al., 2009; Rapport et al., 1948; Zahar et al., 2022), researchers have utilized Trp depletion to explore serotonergic mechanisms (Gibson, 2018). Unsurprisingly, insufficient intake or excessive dietary restriction of Trp leads to a decrease in 5-HT levels in the human brain, resulting in behavioral alterations associated with depression, anxiety, impulsivity, and hyperactivity (Nayak et al., 2019). Raleigh et al. (1988) conducted a study in vervet monkeys (*Cercopithecus aethiops sabaeus*) and unveiled that Trp administration led to alterations in multiple behaviors. Specifically, social grooming, approaching, resting, and eating were increased, while locomoting, avoiding, solitary, and vigilance were decreased (Raleigh et al., 1988). Oral Trp supplementation in male rhesus monkeys showed its potential role in the treatment of self-injurious behavior and its impact on behavior and central serotonin turnover (Weld et al., 1998). Thus, it needs further exploration if the high plasma Trp levels in MFRC-adults affect behavior by influencing plasma 5-HT levels and serotonergic mechanisms.

Previous studies have investigated the role of specific microbial taxa belonging to *Lactococcus, Lactobacillus, Streptococcus, Escherichia coli*, and *Klebsiella* that were able to produce 5-HT by expressing tryptophan synthetase (Gao et al., 2020; O'Mahony et al., 2015). Several bacterial species including *Escherichia coli*, *Peptostreptococcus russellii*, *Clostridium sporogenes*, *Lactobacillus spp* have

been reported to metabolize Trp into various metabolites such as tryptamine, indole, and its derivates including indole-3-aldehyde, indole-3-acetic-acid, and indole-3-propionic acid (Agus et al., 2018). In Chapter III, the microbiome compositions of MFRC-*Mfa* and BTB-*Mff* populations were analyzed using freshly defecated excretion without host's identification. With the significant difference of plasma Trp levels between MFRC-*Mfa* and BTB-*Mff* adults was detected, it becomes unfortunate to not be able to link the Trp levels observed in this chapter and the microbiome composition analyzed in Chapter III. To gain a more comprehensive understanding of the specific associated role of bacterial taxa and Trp metabolism, future research focusing on analyzing microbiome composition and plasma Trp levels collected from the same monkey individuals should be done.

In conclusion, the present study utilizing the HPLC method indicated a significantly higher plasma Trp level in MFRC-*Mfa* adult stone-tool users than the BTB-*Mff* adult non-stone-tool users. This suggested a plausible association between the gut (i.e, diets via the use of stone-tools)-brain (i.e., learning and cognition) axis through Trp levels. Understanding the intricate interplay between Trp, 5-HT, and the gut-brain axis has significant implications in the CNS. Further research in this field may provide insights into potential therapeutic strategies targeting the gut microbiota and serotonin signaling and their underlying mechanisms.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

The gut microbiota and the host have a mutually beneficial relationship in the animal's digestive system. The host provided an environment for the microorganisms, while the microbes aided in the absorption of nutrients for the host (Roberfroid, 1998). The diversity of the gut microbiota is influenced by the interactions and coevolution between the host and its environment. Microbes residing in the mammalian gut played various important roles, including regulating the immune system (Brown et al., 2019; Salzman, 2011), and participating in metabolism-related processes (Janssen & Kersten, 2015). Recent studies have shown that the gut microbiota can be influenced by various factors, including diet, environment, and host genetics (David et al., 2014; Frankel et al., 2019; Karl et al., 2018; Khachatryan et al., 2008; Wu et al., 2011; Zhao et al., 2018). Because these three factors are typically interconnected and had no clear separation, thus they are closely associated with each other (David et al., 2014; Zhao et al., 2018). In addition, the natural environment played crucial roles in the survival and evolution of animal species, and the different habitats imposed selective pressures that driven adaptive changes (Zhang et al., 2016). NHPs had originated from mangrove forests to coastal environments and diversified into woodland, montane, and human settlement (Fooden et al., 1995). In order to thrive in these various geographical environments, NHPs have evolved specialized anatomical features such as distinct stomach structures, lived in distinct habitat preferences, and developed foraging behaviors that allow them to select and extract nutrients from specific food sources (Fleagle, 1989; Milton & May, 1976).

This study surveyed the four populations of two subspecies of free-ranging *M. fascicularis* (*Mff* and *Mfa*) in Thailand, living in two different habitat types (i.e., island and mangrove forest), and the fecal specimens were collected for analyzing the gut microbiota using 16S rRNA gene sequencing on Oxford Nanopore Technologies (Table 3.1; Chapter III). The findings indicated that *Mff* populations exhibited higher bacterial species richness (alpha diversity) in their gut microbiota compared to respective *Mfa* populations living in the same habitat types (Fig. 3.4; Chapter III). This difference is likely attributed to the influence of human provisioning of alternative food sources to mainly the *Mff* populations. These results aligned with a previous study conducted on rhesus macaques, which also suggested that populations receiving human-provided foods tended to have higher microbial richness than their wild counterparts (Chen et al., 2020). Apart from the human food provisioning to the *Mff* populations, the genetic differences between the two subspecies (*Mff* and *Mfa*) (Bunlungsup et al., 2016; Matsudaira et al., 2018; Osada et al., 2021; Phadphon et al., 2022), and the presence of stone-tool use behavior in the *Mfa* and KPE-*Mff* populations may also contribute to these differences, as observed during their foraging behavior.

Among the Old-World monkeys, *Mfa* was reported being the only stone-tool user during foraging for foods. *Mfa* distributed in Myanmar, the Mergui Archipelago, and the Andaman Sea Coast in southwestern Thailand (Fooden, 1995; Luncz et al., 2019; Malaivijitnond et al., 2007). Through mtDNA analysis conducted by Bunlungsup et al. (2016) and Matsudaira et al. (2018), it was revealed that the *Mfa* originated in Myanmar/Bangladesh and then migrated southeastward to southwestern Thailand (Bunlungsup et al., 2016; Fooden, 1995). The natural range of *Mfa* closely

overlapped with that of *Mff*, and the hybrid between the two subspecies has been reported in the peninsula part of Thailand at 8°10' – 12°24'N (Fig. 2.5) (Fooden, 1995). Based on the extensive surveys conducted in the past throughout Thailand covering the distribution range of wild *Mff* populations (Malaivijitnond & Hamada, 2008; Malaivijitnond et al., 2005) and a captive study upon training the *Mff* on stone-setting (Bandini & Tennie, 2018)), however *Mff* were not found to perform stone-tool use behaviors. The stone-tool use behavior had only been reported in the *Mfa* and the *Mfa* x *Mff* hybrids (Bunlungsup et al., 2016; Luncz et al., 2017; Malaivijitnond et al., 2007; Tan, 2017). Thus, the genetic predisposition of *Mfa* was hypothesized to play a critical role in the emergence and development of the stone-tool use behaviors in wild long-tailed macaques (Gumert et al., 2019; Reeves et al., 2023). During my field survey and specimen collection for microbiome analysis at KPE island in Chapter III, I discovered the first *Mff* population that used stone-tool to forage for invertebrate seafood in their natural habitat.

Although the KPE population was surveyed several times in the last decades by other Thai researchers (Suchinda Malaivijitnond, personal communication), however no stone-tool use behavior was seen. The *Mff* subspecies of KPE monkeys were confirmed by distribution, morphological and genetic (mtDNA and *SRY* gene) characteristics (Chapter IV). This discovery opposed the above hypothesis of the *Mfa*'s genetic predisposition on emergence of the stone-tool use behavior in long-tailed macaques. Since this behavior was discovered during the COVID-19 pandemic, it suggests that food scarcity and the need for sustenance may have driven the adaptation to exploit marine food sources. Discounting the emergence of stone-tool use behavior, a potential genetic contribution to the skill of stone-tool manipulation

(or stone-tool use development) in this species is still remained. The stone-tool use behavior observed in the KPE-*Mff* population was hypothesized to have originated from uncoordinated stone throwing, gradually evolving into pound-hammering-like manipulation, which was less proficient than the pound-hammering behaviors reported in the *Mfa* and *Mfa* x *Mff* hybrids (Malaivijitnond et al., 2007; Bunlungsup et al., 2016; Tan, 2017; Luncz et al., 2017). Further extensive studies are needed to determine whether the pound-hammering-like behavior in KPE-*Mff* remains unchanged or progresses to more advanced techniques, such as axe-hammering, as observed in pureblood *Mfa* and *Mfa* x *Mff* hybrids. Consequently, the discovery of the stone-tool use behavior in the KPE-*Mff* population raised several important questions regarding the prevalence of behavior, feeding range, stone manipulation style, stone-tool characteristics, tool-use efficiency, and potential for propagation of this behavior among macaques and between generations. Long-term follow-up of changes of microbiome profile in association with stone-tool use development in each KPE individual should deepen the understanding of the cultural significance and evolutionary trajectory of gut-brain axis in *Mff* subspecies.

The gut microbiota of both *Mff* and *Mfa* populations in this study were predominantly composed of Firmicutes and Bacteroidetes, similar to other NHPs (Amato et al., 2015; Eckburg et al., 2005; Frankel et al., 2019; Hale et al., 2018; Lan et al., 2017; Sawaswong et al., 2021, 2023; Zhao et al., 2018). The relative abundance of Firmicutes also varied between the two *M. fascicularis* subspecies and two habitat types. A higher ratio of Firmicutes to Bacteroidetes was associated with increased dietary energy absorption (Kaakoush, 2015). In this study, the MFRC-*Mfa* population showed reduced microbial diversity resulting in a higher abundance of the

Clostridium sardiniense (Fig. 3.5; Chapter III). These findings are significant for the health of NHPs because reduced diversity in the gut microbiota resulted in fewer microbial metabolic pathways interacting with food items and providing fewer nutritional benefits to the hosts. In other mammalian species, low gut microbial diversity has also been linked to increased susceptibility to opportunistic pathogens (Lozupone et al., 2012). Since the *Oscillibacter valericigenes* and *Faecalibacterium prausnitzii* were notable bacterial species presented in all four populations (Fig. 3.5; Chapter III), it suggests that these bacterial species have significant roles in the health of *M. fascicularis* which were also commonly found in humans (Mondot et al., 2011; Sokol et al., 2008). It also implies a potential shared microbial ecology between the two primate lineages; long-tailed macaques and humans.

The gut-brain axis is a bidirectional communication between the gut and the brain which comprised of CNS, ANS, ENS, and HPA axis (Cryan & Dinan, 2012). The gut-brain axis is communicated and regulated through various pathways, including neural, endocrine, immune, and metabolic (Mayer, 2011). Trp was one of the factors in the gut-brain regulation because of its metabolite, 5-HT (O'Mahony et al., 2015). 5-HT is a crucial neurotransmitter involved in the modulation of emotion, attention, and cognition and exerted its regulatory influence on the gut-brain axis through serotonergic signaling (Kim & Camilleri, 2000). Thus, Trp and 5-HT were selected as representative parameters to infer the communication between the gut and the brain in *M. fascicularis* stone-tool users (MFRC-*Mfa*) and non-stone-tool users (BTB-*Mff*) living in the same habitat type of mangrove forest. Using HPLC technique was unfortunately unable to detect 5-HT levels possibly due to the degradation or low levels in plasma. After statistical analysis, plasma Trp level in the adult MFRC-*Mfa*

stone-tool users were significantly higher than the adult BTB-*Mff* non-stone-tool users. These findings implied that there is a potential link between stone-tool use, plasma Trp levels, and cognitive processes in the MFRC-adults. The high Trp levels in the MFRC-adults reflect the high consumption of Trp-rich foods (O'Mahony et al., 2015), presumably acquired through the stone-tool use. Changes in plasma Trp levels potentially contribute to different cognitive abilities between the two macaque populations. However, it needs further investigation to indicate which specific food types in the MFRC habitat provide a high Trp availability. Gaining insight into these factors will contribute to a more comprehensive understanding of the gut-brain axis, plasma Trp level, and their effect on CNS (learning and cognition) function in association with stone-tool use behavior in NHPs.

In conclusion, this study was to unveil the effects of the environment (habitat type), diet, and host genetics on the gut microbiota by using *M. fascicularis* as animal models. Although it is clearly shown that the diet (acquired via human provision and foraging using either stone-tool or non-stone-tool) is a predominant factor that affects the microbiome profile in macaques, the influence of the host genetic and the habitat types could not be ruled out. As the gut-brain axis is a bi-directional communication, it needs to investigate further if changes in plasma Trp levels via different food consumptions will affect the brain development and the learning and cognitive ability in stone-tool use macaques. Hopefully, the preliminary results in this thesis can ignite and inspire other researchers to develop the following important research questions in the microbiome field.

Recommendations

1. This study did not identify and collect information of food types consumed by monkeys all year round, and the results might not completely reflect the association between the microbiome proportion and the diets. Therefore, it is recommended to collect data on food types, especially natural foods, which are seasonally available.
2. This study was conducted at the population level; however, each animal should have a different food preference. Therefore, it is recommended to collect microbiome data (composition and diversity) and consumption of food types at the individual level.
3. To clearly interpret the association between the gut-brain axis (gut microbiota and Trp metabolism), it is suggested that fecal (for microbiome study) and plasma (for Trp level) specimens should be collected from the same individuals. In this study, the fecal specimens were acquired from the freshly defecated excretion, and later the plasma specimens were collected from animals after capturing and anesthetization.
4. An appropriate specimen such as platelet-rich plasma is recommended for the detection of 5-HT levels.

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