Received: 09 February 2024 Revised: 07 May 2024 Accepted: 20 May 2024
DOI: https://doi.org/10.29244/primatology.3.01.1-17 E-ISSN: 2964-5441

RESEARCH ARTICLE

INAJP INDONESIAN JOURNAL OF PRIMATOLOGY

Isolation and Biochemical Characterization of Aerobic Gut Bacteria from Long-Tailed Macaques on Tinjil Island, Indonesia

Anggitha Tambunan[1], Puji Rianti[1,2*], Jepri Agung Priyanto[1], Huda Shalahudin Darusman[2,3], Randall C. Kyes[2,4]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University. Jl. Agatis, Kampus IPB Dramaga, Bogor, Indonesia
[2] Primate Research Center, Institute of Research and Community Services, IPB University, Jalan Lodaya II No. 5, Bogor, Indonesia
[3] School of Veterinary Medicine and Biomedical Sciences, IPB University. Jl. Agatis, Kampus IPB Dramaga, Bogor, Indonesia
[4] Departments of Psychology, Global Health, and Anthropology, Center for Global Field Study, and Washington National Primate Research Center, University of Washington. 3980 15th Ave NE Seattle, Washington 98195, USA

Abstract
Long-tailed macaques (*Macaca fascicularis*; LTMs) are a commonly used non-human primate model in medical research due to their physiological and genetic similarity to humans. Maintaining the balance of gut microbiota in LTMs is crucial to mitigate the risk of dysbiosis-related diseases. Thus, the aim of this study was to isolate bacteria from semi-wild LTMs inhabiting Tinjil Island in order to assess the diversity of their gut microbiota. Fecal samples from four semi-wild LTMs were serially diluted and planted onto nutrient agar medium to enumerate bacteria via Total Plate Count (TPC). Bergey's Manual Determinative of Bacteriology was used for bacterial identification, utilizing morphological and biochemical characteristics. The average total viable bacterial count obtained was 1.86 x 10^9 CFU/g. Aerobic isolation of bacteria from all samples yielded 19 isolates of gram-positive bacteria, including six putative species of *Staphylococcus* sp., three *Bacillus* sp., four *Micrococcus* sp., and six *Corynebacterium* sp. Overall, the isolation of cultivable fecal microbiota from the four LTM fecal samples from Tinjil Island has provided initial insight into the composition of the macaques' gut microbiota, albeit through limited analytical methods reliant on culture-dependent approaches.

Key words: culture-method, fecal samples, gut microbiota, *Macaca fascicularis*.

1. Introduction

Long-tailed macaques (*Macaca fascicularis; LTMs*), also known as crab-eating or cynomolgus monkeys, represent a non-human primate (NHP) species within the subfamily Cercopithecinae and family Cercopithecidae. In 2022, the International Union for Conservation of Nature (IUCN) declared LTMs as an Endangered Species (Hansen *et al.* 2022). However, the assessment data supporting this decision have been subject to scrutiny, particularly with regard to their extinction probabilities in the wild (Hilborn and Smith 2024). LTMs are found throughout a number of countries in Southeast Asia and are reported to have the second-largest distribution of all macaque species (Liedigk *et al.* 2015). They have a lifespan that can extend up to 25-30 years (Li *et al.* 2023), and given their physiological and genetic similarities to humans, LTMs are frequently used as animal models in medical research (Stevison and Kohn 2008).

The significant role of this species as an animal model has led to widespread breeding efforts, both in captivity and in natural settings, such as in Tinjil Island. Tinjil Island serves as a natural habitat breeding facility of the Primate Research Center, IPB University, located in Banten Province, Indonesia (Pamungkas *et al.* 1994). Originally introduced to this forested island in 1988 (Kyes 1993), the LTMs are free-ranging, subsisting on natural food sources such as fruits, leaves, insects, small lizards, and crabs. Additionally, modest supplemental provisions, such as bananas and corn, are provided to the macaque groups on the island. In captive environments, the LTMs' diet typically consists of commercial monkey biscuits or monkey chow, with limited portions of seasonal fruits (Takenaka *et al.* 2000). Differences in diet between captive and wild environments reportedly influence changes in the composition of LTMs' gut microbiota (Boonkusol *et al.* 2020 Frankel *et al.* 2019; Grant *et al.* 2019; Wills *et al.* 2022).

The diversity of gut microbiota is influenced by a variety of factors, including intrinsic elements such as the age and sex of host species, as well as extrinsic factors like environment and diets (Ying *et al.* 2022). Diet, in particular, plays a crucial role in shaping gut microbiota diversity, as demonstrated by significant alterations in gut microbiota diversity within just a year of transferring LTMs from their natural habitat to captivity (Sawaswong *et al.* 2023). In primates, the gut microbiota comprises bacteria, fungi, protozoa, viruses, and archaea. These microorganisms play essential roles in metabolizing complex nutrients (Chen *et al.* 2017), maintaining immune system function (Muegge *et al.* 2011), and regulating hormone activity (Martin *et al.* 2019). Additionally, the gut microbiota serves as a secondary neural network, facilitating bidirectional communication between the nervous system and gut microbiota, known as the Microbiota-Gut-Brain-Axis

*Coresponding author
Email Address : pujirianti@apps.ipb.ac.id

(MGBA) (Carabotti *et al.* 2015). This communication occurs through various mechanisms, including the stimulation of cytokine expression, production of microbial metabolites such as short-chain fatty acids (SCFAs), and tryptophan metabolism (Carbia *et al.* 2023). Moreover, the gut microbiota contributes to the synthesis of neurotransmitters like serotonin which influences gastrointestinal motility and homeostasis (Yano *et al.* 2015), and γ-aminobutyric acid (GABA) which modulates blood pressure and immune function (Pokusaeva *et al.* 2017).

Maintaining the balance of bacteria in the intestine is crucial for healthy bodily function. Dysbiosis, characterized by an imbalance in gut microbiota composition, can lead to the development of idiopathic chronic diarrhea (ICD) in LTMs (Koo *et al.* 2020), immune imbalance in rhesus macaques (Li *et al*. 2020), and has been associated with depressive-like behavior in LTMs serving as a model animal (Wu *et al*. 2022). Consequently, understanding the gut microbiota composition becomes pivotal in assessing LTM health. Despite research efforts focusing on various aspects of LTMs on Tinjil Island, including population dynamics (Perwitasari-Farajallah *et al.* 2023a), behavior (Hasanah *et al.* 2022), and feeding ecology (Perwitasari-Farajallah *et al.* 2023b), studies on gut microbiota composition of semi-wild LTMs have not yet been conducted. Hence, the aim of this study was to assess intestinal bacteria from LTMs on Tinjil Island by isolating and characterizing gut bacteria from their fecal samples using a culture-dependent method. This research provides preliminary insight into the diversity of gut bacteria in semi-wild LTMs from Tinjil Island.

2. Materials and Methods

2.1. Fecal Sample Collection

In July 2022, we collected a single fecal sample, noninvasively, from four semi-wild LTMs on Tinjil Island, Banten, Primate Research Center, IPB University (Figure 1), with approval from the IPB University Animal Care and Use Committee of Ethics (ACUC-IPB University; no. IPB PRC-19-A012). A sterile wooden stick was used to collect freshly expelled fecal samples, which were then placed into Falcon tubes and refrigerated to preserve their condition for subsequent analysis. Samples were collected from the inner part of the feces, ensuring that they were not exposed to the ground or air.

2.2. Total Plate Count (TPC)

The TPC method was employed to estimate the population of microorganisms within the growth media by enumerating each bacterial colony. One gram of each LTM fecal sample was homogenized in 9 mL of 0.85% NaCl solution, followed by serial dilution eight times. Subsequently, 0.1 mL of the diluent from 10^{-4} to 10^{-8} serial dilution was spread onto nutrient agar (NA) medium and then incubated aerobically at room temperature (25±2°C for 24-48 hours, with modifications adapted from (Prats *et al.* 2008). This procedure was used to quantify bacterial colonies on each agar plate using TPC, resulting in colony-forming units (CFU/g). Enumeration of the bacterial colonies on each plate was exclusively conducted at dilutions where the total colony count ranged from 30 to 300 bacterial colonies (Zuberer 1994). Colonies numbering less than 30 were classified as "too few to count" (TFTC), while those exceeding 300 were considered "too numerous to count" (TNTC).

2.3. Bacterial Isolates Purification

We observed the bacterial colonies on each sample, observing variations in morphology, including form, margin, texture, color, transparency, and elevation, before isolating purified bacteria. We then isolated each colony with distinctive features using the quadrants streak method into the NA plate medium to attain a pure single bacterial colony. The isolates were placed into a refrigerator for further bacteria stain after incubation for about 24-48 hours at 25±2°C.

2.4. Gram Stain and Biochemical Test

We classified bacteria into two groups using the Gram stain method: Gram-positive and Gram-negative bacteria. Gram-positive bacteria exhibit a purple or blue color when viewed under the microscope, while Gram-negative bacteria appear red (Smith and Hussey 2020). We considered the different bacteria cells' shapes and Gram stain status to move on to the specific biochemical test pathway.

We used biochemical tests to discern various bacteria based on their reactions with different biochemical compounds. Each isolate was identified through a series of biochemical tests following Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994). Specific biochemical test flowcharts were employed for each bacterial isolate depending on the type of bacteria revealed by Gram staining the shape of the cell. In this study, we used two biochemical test flowcharts: The Gram-positive cocci flowchart and the Gram-positive bacilli catalase flowchart (Supplementary Material Figure S1).

2.4.1. Catalase Test for Gram-positive cocci and Gram-positive bacilli

We conducted the slide or drop catalase test to detect the presence of the enzyme catalase in bacteria (Reiner 2013). A positive result was immediately observed when bubbles formed on the slide. Positive results from the catalase test in the Gram-positive cocci group proceeded to the mannitol fermentation test stage (Hanson 2008). Conversely, positive results from the catalase test in the Gram-positive bacilli group led to the starch hydrolysis test (Hussey 2008) with modifications. Colonies resistant to acid decolorization during stain procedures prompted further testing using the acid-fast stain protocol (Hussey and Zayaitz 2013) employing carbolfuchsin as the primary stain, methylene blue as the counterstain, and acid-alcohol as the decolorizing solvent. The Voges-Proskauer (VP) test determined the organism's ability to produce acetylmethylcarbinol from glucose

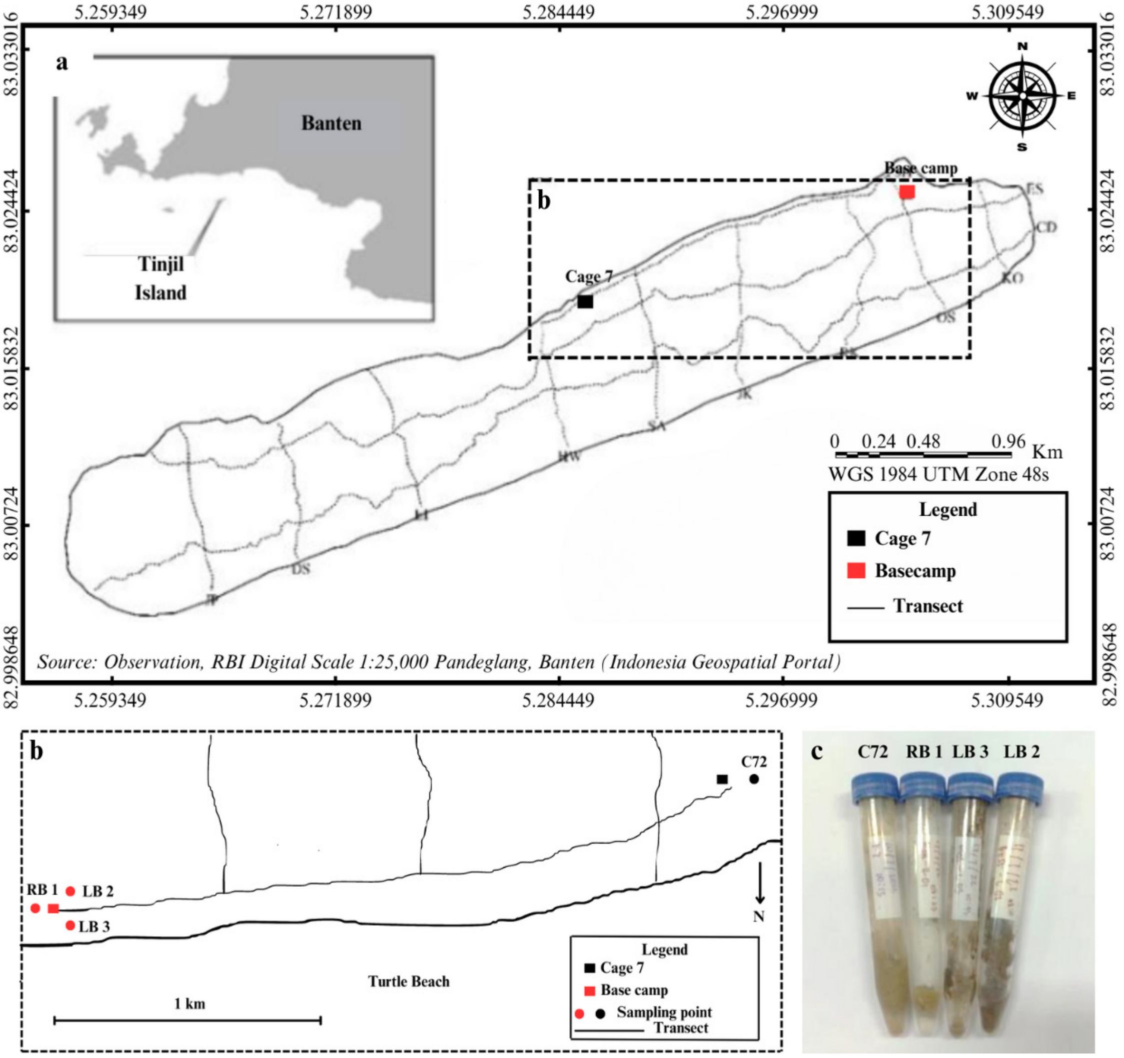

Figure 1. Fecal sampling locations of the long-tailed macaques on Tinjil Island. a) Map of Tinjil Island (modified from Perwitasari-Farajallah *et al.* 2023a); b) Location of fecal sampling points at Basecamp and Cage 7; c) Four fecal samples, one each from four long-tailed macaques: RB1 (Right Basecamp Sample), LB2 and LB3 (Left Basecamp Sample), C72 (Cage 7 Sample).

fermentation (following Mcdevitt's protocol; Mcdevitt 2009) by preparing Methyl Red-Voges Proskauer (MRVP) broth as per the manufacturer's instructions. For the salt tolerance test, we utilized nutrient broth (NB) supplemented with sodium chloride to achieve a salt concentration of 6.5% (6.5 g NaCl per 100 mL NB media) (Bruins *et al*. 2007). Inoculum from a pure culture (aged 18-24 hours) was transferred to sterile 6.5% NaCl broth and incubated at 35-37°C for 24 hours. The presence of turbidity indicated a positive test result.

2.5. Hemolysis for All the Isolates

We conducted the hemolysis test by inoculating a colony from a fresh (16-18 hours), pure culture onto blood agar plates (BAP) and subsequently incubating them at 35±2°C for approximately 18-24 hours. Bacterial hemolytic reactions are categorized into three types: Beta hemolysis (β), alpha hemolysis (α), and gamma hemolysis (γ) (Buxton 2016). We conducted our observation with the light shining from behind the plate to interpret the hemolytic reaction of each bacterial streak accurately. Beta hemolysis manifests as a complete lysis of red blood cells, appearing as a clear zone or transparency surrounding the colony. A green or brown discoloration in the medium characterizes alpha hemolysis. Finally, gamma hemolysis is indicated by the absence of a clear zone surrounding the colony on the medium.

3. Results

3.1. Total Viable Bacteria in LTMs Fecal Samples

According to the total plate count method, the average of viable bacteria found in the fecal samples from the four LTMs was 1.86 x 10^9 CFU/g (Supplementary Material Table S1). However, each sample exhibited varying quantities of bacterial colonies. Sample C72 displayed the highest number of viable bacteria, whereas Sample RB1 exhibited the lowest.

3.2. Bacterial Colony Morphology

Various morphological characteristics were observed among different bacterial colonies grown in each sample (Supplementary Material Table S2,

Figure 2). Sample LB3 exhibited the highest number of bacterial isolates, totaling six isolates, while sample C72 had the fewest, with only three isolates. Among the three bacterial isolates from sample C72, only the color differed, while other characteristics remained the same. Additionally, two bacterial isolates with identical colony morphology were identified in sample C72, suggesting they were the same type of bacteria. The colony morphology of bacterial isolates from samples RB1 and RB2 varied significantly, indicating the presence of different types within these samples. Similarly, sample LB2 contained six isolates, two of which shared similar macroscopic characteristics, namely isolates LB3.1 and LB3.4. Furthermore, isolates with identical morphological characteristics were found across samples, such as isolate RB1.4 with isolate LB3.3 and isolate RB1.1 with isolate LB2.3.

3.3. Bacterial Cell Morphology

The characteristics of bacterial cell morphology, including shape, cell arrangement, and Gram group, were assessed (Supplementary Material Table S3, Figure 3). All bacterial isolates were identified as Gram-positive bacteria, predominantly cocci-shaped, with a smaller proportion being bacilli-shaped. Isolate RB1.4 and Isolate LB3.3 exhibited coccobacilli-shaped morphology, intermediate between cocci and bacilli shapes. The arrangement of the bacterial cells varied within each group of Gram-positive cocci and Gram-positive bacilli, with dominance determined through microscopic observation.

3.4. Bacterial Identification with Biochemical Tests

Biochemical tests were conducted for the identification of Gram-positive cocci and Gram-positive bacilli, as detailed in Tables 1 and 2, respectively. Based on biochemical test results, only two groups of putative bacterial genera were identified: *Staphylococcus* and *Micrococcus* for the Gram-positive cocci group (Table 1). Isolates C72.2 and C72.3 were identified as *Staphylococcus aureus,* a conclusion supported by their macroscopic colony characteristics (Supplementary Material Table S2). Four bacterial isolates that could not ferment mannitol (indicated by the green OF media color) but exhibited yellow colonies were suspected to be *Micrococcus luteus* bacteria (Supplementary Material Table S4).

Overall, bacterial isolates suspected to be *Staphylococcus* spp. (excluding *Staphylococcus aureus*) shared similar biochemical test characteristics. Similarly, bacterial isolates presumed to be *Micrococcus luteus* species displayed a consistent inability to ferment mannitol. However, a notable difference was observed in colony pigmentation, with suspected *Micrococcus luteus* isolates exhibiting

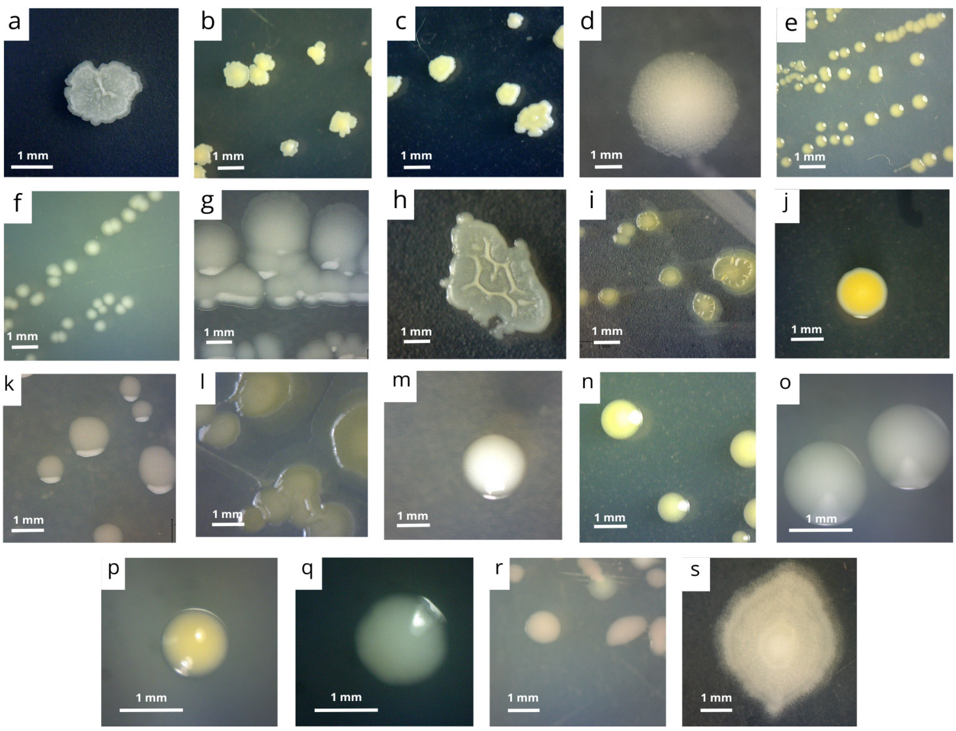

Figure 2. Bacterial colonies isolated from fecal samples of wild long-tailed macaques on Tinjil Island. (a) C72.1 (Cage 7 Sample 2 Isolate 1), (b) C72.2 (Cage 7 Sample 2 Isolate 2), (c) C72.3 (Cage 7 Sample 2 Isolate 3), (d) LB2.1 (Left Basecamp Sample 2 Isolate 1), (e) LB2.2 (Left Basecamp Sample 2 Isolate 2), (f) LB2.3 (Left Basecamp Sample 2 Isolate 3), (g) LB2.4 (Left Basecamp Sample 2 Isolate 4), (h) LB2.5 (Left Basecamp Sample 2 Isolate 5), (i) LB3.1 (Left Basecamp Sample 3 Isolate 1), (j) LB3.2 (Left Basecamp Sample 3 Isolate 2), (k) LB3.3 (Left Basecamp Sample 3 Isolate 3), (l) LB3.4 (Left Basecamp Sample 3 Isolate 4), (m) LB3.5 (Left Basecamp Sample 3 Isolate 5), (n) LB3.6 (Left Basecamp Sample 3 Isolate 6), (o) RB1.1 (Right Basecamp Sample 1 Isolate 1), (p) RB1.2 (Right Basecamp Sample 1 Isolate 2), (q) RB1.3 (Right Basecamp Sample 1 Isolate 3), (r) RB1.4 (Right Basecamp Sample 1 Isolate 4), (s) RB1.5 (Right Basecamp Sample 1 Isolate 5). White line on under leftside of every part of figure is a scale.

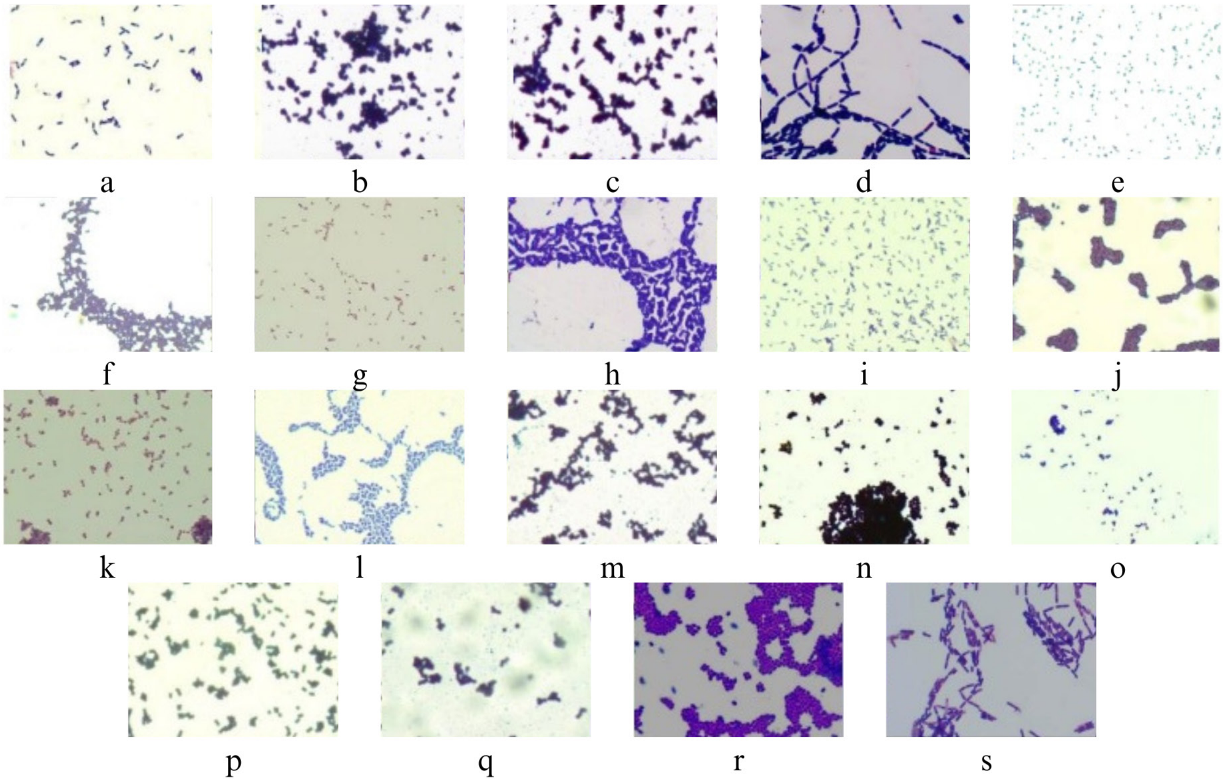

Figure 3 Positive-gram bacteria isolated from fecal samples of wild long-tailed macaques on Tinjil Island (a) C72.1 (Cage 7 Sample 2 Isolate 1), (b) C72.2 (Cage 7 Sample 2 Isolate 2), (c) C72.3 (Cage 7 Sample 2 Isolate 3), (d) LB2.1 (Left Basecamp Sample 2 Isolate 1), (e) LB2.2 (Left Basecamp Sample 2 Isolate 2), (f) LB2.3 (Left Basecamp Sample 2 Isolate 3), (g) LB2.4 (Left Basecamp Sample 2 Isolate 4), (h) LB2.5 (Left Basecamp Sample 2 Isolate 5), (i) LB3.1 (Left Basecamp Sample 3 Isolate 1), (j) LB3.2 (Left Basecamp Sample 3 Isolate 2), (k) LB3.3 (Left Basecamp Sample 3 Isolate 3), (l) LB3.4 (Left Basecamp Sample 3 Isolate 4), (m) LB3.5 (Left Basecamp Sample 3 Isolate 5), (n) LB3.6 (Left Basecamp Sample 3 Isolate 6), (o) RB1.1 (Right Basecamp Sample 1 Isolate 1), (p) RB1.2 (Right Basecamp Sample 1 Isolate 2), (q) RB1.3 (Right Basecamp Sample 1 Isolate 3), (r) RB1.4 (Right Basecamp Sample 1 Isolate 4), (s) RB1.5 (Right Basecamp Sample 1 Isolate 5).

yellow-pigmented colonies while those of suspected *Staphylococcus* spp. (except *Staphylococcus aureus*) tended to have white colonies (Supplementary Material Table S4).

Based on the biochemical tests conducted on nine isolates of Gram-positive bacilli, two bacterial genera were identified: *Bacillus* and *Corynebacterium,* encompassing three putative species, namely *Bacillus cereus, Corynebacterium xerosis,* and *Corynebacterium kutscheri* (see Table 2, Figure 3, Supplementary Material Table S4, Table S5, Table S6). The endospore-forming bacterial isolates (LB2.1 and RB1.5) exhibited consistent results across all biochemical tests, indicating the presence of catalase and amylase enzymes (Figure 4), as well as acetoin production, as indicated by a positive VP test result.

Conversely, endospore-forming bacterial isolate C72.1 lacked the enzyme necessary for sugar breakdown into acetoin (negative VP test), differing from the other two endospore-forming isolates (Supplementary Material Table S6). Isolate C72.1 was categorized as swollen cells due to the larger diameter of the spore compared to vegetative cells. Additionally, isolate C72.1 tested negative in the NaCl 0.65% test, indicating susceptibility to media conditions with NaCl 6.5%. Although two presumed bacterial species (*B. pantothenic* and *B. circulans*) were identified at this stage, the arabinose fermentation test could not be conducted, thus halting future identification.

Other bacterial isolates lacking endospore underwent shorter biochemical test pathways, including acid-fast staining, catalase test, and starch hydrolysis. Variations in starch hydrolysis test results distinguished six isolates into two different *Corynebacterium* species. Isolates forming clear zones (LB2.4, LB2.5, LB3.1, LB3.4) were identified as *Corynebacterium kutscheri*, while those lacking clear zones (LB3.3 and RB1.4) were identified as *Corynebacterium xerosis* (Figure 4).

Hemolytic activity tests were conducted on all bacterial isolates, revealing various hemolytic types (Supplementary Material Table S6; Figure 4). Beta-hemolytic bacterial isolates formed clear zones around isolates, exemplified by isolates RB1.5, C72.1, and LB2.1 (Figure 4). Alpha-hemolytic bacteria were characterized by slightly darker streaks in the middle of quadrants, as observed in isolates LB3.1 and LB2.5 (Figure 4). Gamma hemolytic bacterial isolates exhibited no color change or alteration in the area surrounding the bacterial colony, as seen in LB3.5 and RB1.3. *Bacillus* isolates displayed beta-hemolytic activity, while other isolates, such as *Staphylococcus*, *Corynebacterium*, and *Micrococcus*, exhibited varying hemolytic patterns.

Tabel 1. Biochemical tests on bacterial isolates of Gram-positive cocci

No	Isolate code	Biochemical test				Putative species
		Ca	M	Y	G	
1	C72.2	+	+	+	x	*Staphylococcus aureus*
2	C72.3	+	+	+	x	*Staphylococcus aureus*
3	LB2.2	+	-	+	-	*Micrococcus luteus*
4	LB2.3	+	-	-	x	*Staphylococcus* sp.
5	LB3.2	+	-	+	-	*Micrococcus luteus*
6	LB3.5	+	-	-	x	*Staphylococcus* sp.
7	LB3.6	+	-	+	-	*Micrococcus luteus*
8	RB1.1	+	-	-	x	*Staphylococcus* sp.
9	RB1.2	+	-	+	-	*Micrococcus luteus*
10	RB1.3	+	-	-	x	*Staphylococcus* sp.

Isolate code represents bacterial isolates obtained from fecal samples of long-tailed macaques on Tinjil Island, including C72.2 (Cage 7 Sample 2 Isolate 2), C72.3 (Cage 7 Sample 2 Isolate 3), LB2.2 (Left Basecamp Sample 2 Isolate 2), LB2.3 (Left Basecamp Sample 2 Isolate 3), LB3.2 (Left Basecamp Sample 3 Isolate 2), LB3.5 (Left Basecamp Sample 3 Isolate 5), LB3.6 (Left Basecamp Sample 3 Isolate 6), RB1.1 (Right Basecamp Sample 1 Isolate 1), RB1.2 (Right Basecamp Sample 1 Isolate 2), RB1.3 (Right Basecamp Sample 1 Isolate 3). Types of biochemical tests: Ca (Catalase: (+) bubbles, (-) no bubbles); M (Mannitol: (+) yellow and bubbles in durham tube, (-) green); Y (Yellow pigment); G (Glucose: (+) yellow and bubbles in durham tube, (-) green). "x" indicates Not Tested.

Table 2. Biochemical tests on bacterial isolates of Gram-positive bacilli

No	Isolate code	Biochemical test								Putative species
		E	Af	Ca	S	VP	Cd	Mo	Na	
1	C72.1	+	x	+	+	-	+	x	-	*Bacillus* sp.
2	LB2.1	+	x	+	+	+	+	+	x	*Bacillus cereus*
3	LB2.4	-	-	+	+	x	+	x	x	*Corynebacterium kutsceri*
4	LB2.5	-	-	+	+	x	+	x	x	*Corynebacterium kutsceri*
5	LB3.1	-	-	+	+	x	+	x	x	*Corynebacterium kutsceri*
6	LB3.3	-	-	+	-	x	+	x	x	*Corynebacterium xerosis*
7	LB3.4	-	-	+	+	x	+	x	x	*Corynebacterium kutsceri*
8	RB1.4	-	-	+	-	x	+	x	x	*Corynebacterium xerosis*
9	RB1.5	+	x	+	+	+	+	+	x	*Bacillus cereus*

Isolate code represents bacterial isolates obtained from fecal samples of long-tailed macaques on Tinjil Island, including C72.1 (Cage 7 Sample 2 Isolate 1), LB2.1 (Left Basecamp Sample 2 Isolate 1), LB2.4 (Left Basecamp Sample 2 Isolate 4), LB2.5 (Left Basecamp Sample 2 Isolate 5), LB3.1 (Left Basecamp Sample 3 Isolate 1), LB3.3 (Left Basecamp Sample 3 Isolate 3), LB3.4 (Left Basecamp Sample 3 Isolate 4), RB1.4 (Right Basecamp Sample 1 Isolate 4), RB1.5 (Right Basecamp Sample 1 Isolate 5). Type of biochemical tests E (Endospore stain: (+) endospore-forming, (-) non-endospore forming), Af (Acid-fast: (+) red cells, (-) blue cell), Ca (Catalase (+) bubbles, (-) no bubbles), S (Starch hydrolysis: (+) clear zone, (-) no clear zone), VP (Voges-Proskauer: (+) red, (-) orange/brown), Cd (Cell diameter: (+) Cd > 1 um, (-) Cd < 1 um), M (Motility test: (+) motile, (-) nonmotile), Na (NaCl 6.5%: (+) cloudy, (-) almost clear/same with negative control). "x" indicates Not Tested.

4. Discussion

This study presents the findings from the isolation of bacteria from fecal samples of four semi-wild LTMs on Tinjil island. A total of 19 putative species of bacteria were identified, including three species of *Bacillus* spp., six of *Staphylococcus* spp. (belonging to the phylum Actinomycetota/Actinobacteria; Gao and Gupta 2012), four of *Micrococcus* spp., and six of *Corynebacterium* spp. (belong to phylum Bacilliota/ Firmicutes; Ludwig *et al.* 2009). Typically, the predominant bacterial phyla in the gut of NHPs include Firmicutes, Bacteroidetes, Proteobacteria, and others (Gogarten *et al.* 2018). Notably, Firmicutes, Bacteriodetes, Proteobacteria, and Tenericutes are highly common in LTMs (Grant *et al.* 2019; Nagpal *et al.* 2018; Sawaswong *et al.* 2021). These findings align with previous studies employing similar methodologies in baboons (Lugano *et al.* 2018). Subsequently, based on the intestinal oxygen concentration and bacterial types, it can be inferred that bacterial genera such as *Bacillus* (Toerien 1967), *Staphylococcus* and *Micrococcus* (Kloos and Musselwhite 1975), and *Corynebacterium* (Nishimura *et al.* 2011) can survive in the intestine under suitable oxygen concentrations conducive to the growth of these types of bacteria.

The *Bacillus* species identified in our study encompassed two putative species: *Bacillus cereus* and *Bacillus* spp. *Bacillus cereus* is known as an emetic and enterotoxin-producing bacterium capable of causing diarrhea (Benedict *et al.* 1993) and has been

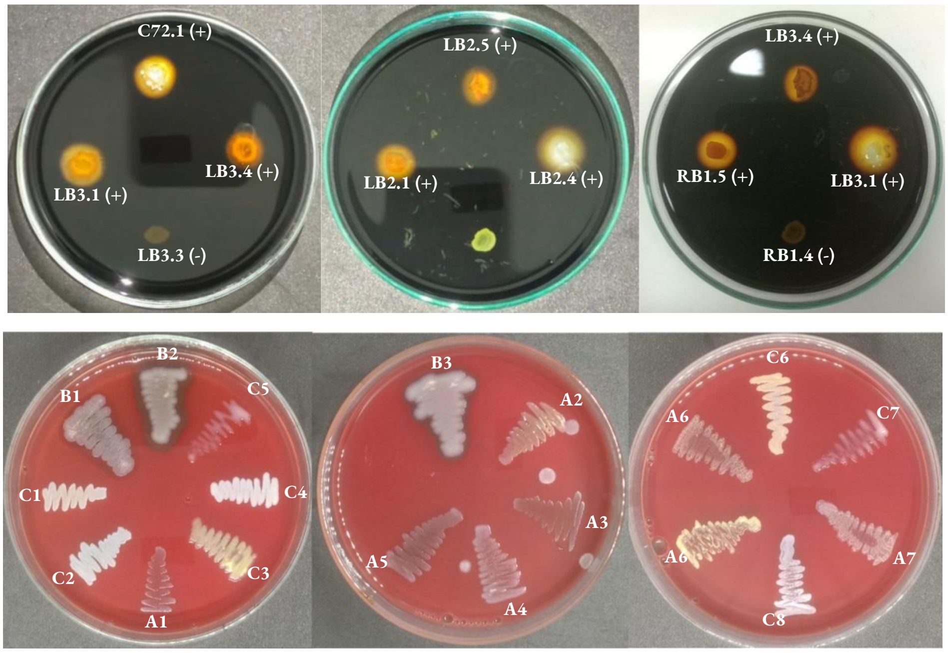

Figure 4 Biochemical tests including starch hydrolysis test and hemolytic test. (Above) Results of starch hydrolysis test for selected isolates: C72.1 (Cage 7 Sample 2 Isolate 1), LB2.1 (Left Basecamp Sample 2 Isolate 1), LB2.4 (Left Basecamp Sample 2 Isolate 4), LB2.5 (Left Basecamp Sample 2 Isolate 5), LB3.1 (Left Basecamp Sample 3 Isolate 1), LB3.3 (Left Basecamp Sample 3 Isolate 3), LB3.4 (Left Basecamp Sample 3 Isolate 4), RB1.4 (Right Basecamp Sample 1 Isolate 4), RB1.5 (Right Basecamp Sample 1 Isolate 5). (Below) Hemolytic activity of bacterial isolates from fecal samples of long-tailed macaques on Tinjil Island, categorized as (A) α-hemolytic, (B) β-hemolytic, and (C) γ-hemolytic). B1 (C72.1/Cage 7 Sample 2 Isolate 1), C1 (C72.2/Cage 7 Sample 2 Isolate 2), C2 (C72.3/Cage 7 Sample 2 Isolate 3), B3 (LB2.1/Left Basecamp Sample 2 Isolate 1), A2 (LB2.2/Left Basecamp Sample 2 Isolate 2), A3 (LB2.3/Left Basecamp Sample 2 Isolate 3), A4 (LB2.4/ Left Basecamp Sample 2 Isolate 4), A5 (LB2.5/Left Basecamp Sample 2 Isolate 5), A6 (LB3.1/Left Basecamp Sample 3 Isolate 1), C6 (LB3.2/Left Basecamp Sample 3 Isolate 2), C7 (LB3.3/Left Basecamp Sample 3 Isolate 3), A7 (LB3.4/Left Basecamp Sample 3 Isolate 4), C8 (LB3.5/Left Basecamp Sample 3 Isolate 5), A8 (LB3.6/Left Basecamp Sample 3 Isolate 6), A1 (RB1.1/Right Basecamp Sample 1 Isolate 1), C3 (RB1.2 /Right Basecamp Sample 1 Isolate 2), C4 (RB1.3/Right Basecamp Sample 1 Isolate).

characterized as a beta-hemolytic (Dabiré *et al.* 2022). *Bacillus* spp. is found in various habitats, including the gut of various insects and animals (Hong *et al.* 2009), and generally exhibits the ability to hydrolyze starch and protein in anaerobic digesters (Toerien 1967). Consequently, *Bacillus* spp. are used in numerous medical, pharmaceutical, agricultural, and industrial processes due to their broad range of physiological characteristics, including the production of enzymes such as amylase (Luang-In *et al*. 2019) and antibiotics (Beneduzi *et al.* 2012). While certain species of *Bacillus*, notably *Bacillus cereus*, are known to be occasional pathogens of humans and livestock, most *Bacillus* spp. are harmless saprophytes (Turnbull *et al*. 2002). In the case of semi-wild LTMs, their diet may consist of plants/fruits, insects, and soil. Spores from *Bacillus cereus* are known to proliferate in the intestines of insects (Margulis *et al.* 1998), which may then be ingested by LTMs, ultimately resulting in the colonization of these bacteria in their intestines. Although one of the putative bacterial species of *Bacillus* identified in this study was classified as pathogenic, there is a possibility that *Bacillus* spp. represent beneficial species of *Bacillus*, such as *Bacillus coagulans*. This speculation arises because all identified bacterial species are still categorized as putative. Furthermore, *Bacillus coagulans* can serve as a probiotic, alleviating clinical symptoms such as bloating, vomiting, diarrhea, and abdominal pain in patients with irritable bowel syndrome (Majeed *et al.* 2016).

The only putative species identified within the *Staphylococcus* genera were *Staphylococcus aureus* and *Staphylococcus* spp. *Staphylococcus* is known to inhabit various environments, including the air (Solberg 2000), animal skins (squirrel, monkey, and sheep) (Kloos *et al.* 1976), and human skin (Raineri *et al.* 2022). *Staphylococcus aureus* is the causative agent of food poisoning, one of the most common foodborne illnesses that can induce nausea (vomiting) and diarrhea (Abril *et al.* 2020). However, the presence of some putative bacterial species in these semi-wild LTM samples does not necessarily imply that the conditions of the LTMs on Tinjil Island are unhealthy or that they are suffering from diarrhea. The results obtained in this study do not permit a full assessment of the complete condition of the gut microbiota of the LTMs on Tinjil with respect to their health, including whether they are experiencing dysbiosis. Nevertheless, direct field observations indicate that the LTMs did not suffer from diarrhea, as evidenced by the stool condition observed during fecal sampling, which exhibited a normal texture (soft and well-formed in consistency).

In addition to *Staphylococcus*, it appears that *Micrococcus* is also a genus of microbes commonly found on the skin of animals, in the air, within the inner tissues of plants, in soil, and among several fish species (Lee *et al*. 2022). The only putative species identified in this study, namely *Micrococcus luteus*, is categorized as human-commensal and non-pathogenic bacterial species (Albertson *et al.* 1978). This study also identified other putative bacterial species, namely *Corynebacterium xerosis* and *Corynebacterium kutscheri*. *Corynebacterium xerosis* is a commensal organism typically present on the skin and mucous membranes of humans and animals (Vela *et al.* 2006), while *Corynebacterium kutscheri* has been described as a commensal bacterium in mice, rats, and voles (Holmes and Korman 2007). The identified putative bacterial species obtained in this study exhibit a range of characteristics, being either non-pathogenic, pathogenic, or commensal to their host. However, only a few (five putative species) could be identified up to the species level in this study.

The limited number of putative bacterial species obtained in this study can be attributed to the small number of bacterial isolates per sample. Among the four fecal samples analyzed, C72 exhibited the lowest bacterial isolates compared to LB2, LB3, and RB1. This scarcity of bacterial isolates in C72 occurred because there were only very few distinct bacterial colonies when isolating bacteria from a solid medium. Identifying certain bacteria enables the discrimination between different bacterial species based on their morphology, arrangement, and consequent colony patterns (Badieyan *et al.* 2018).

The incubation technique used in this study also likely played a role in affecting the lower diversity of isolated bacteria in this study. All procedures, including sampling collection, preparation, and isolation, were conducted aerobically without implementing anaerobic conditions. Consequently, only aerobic or facultative anaerobic bacteria could thrive, while obligate anaerobic bacteria could not. Ideally, bacterial isolation should encompass both aerobic and anaerobic conditions to facilitate the growth of a wider range of bacteria. Moreover, not only should fecal sample collection and storage be performed anaerobically in the laboratory, but it is imperative due to the dominant presence of anaerobic bacteria, including Bacteroidetes, Firmicutes, and Proteobacteria, comprising over 95% of rectal microbiota in rhesus and LTM macaques (Cui *et al.* 2019). Therefore, exposure to oxygen should be carefully considered as an influential factor.

The isolation of cultivable fecal microbiota from semi-wild LTMs on Tinjil Island has provided a basic overview and preliminary baseline data of gut bacterial diversity through culture-dependent methods. However, studies examining intestinal bacteria diversity through morphological and biochemical analysis have inherent limitations compared to molecular analysis. Non-molecular methods can only culture bacteria capable of growth in vitro conditions, thus restricting the range of detectable bacteria (Hayashi *et al.* 2022). Therefore, molecular approaches, such as metagenomic analysis, are essential for a comprehensive understanding of gut microbiota profiles. Molecular analysis offers increased sensitivity and accuracy in identifying bacterial species compared to culture-dependent methods (McKenna *et al.* 2008; Rhoads *et al.* 2012).

Nevertheless, intestinal bacteria play a crucial role as key regulators of digestion throughout the gastrointestinal tract (Bornbusch *et al*. 2023; Rinninella *et al.* 2019; Turnbaugh and Gordon 2009). Various factors influence the diversity and composition of gut microbiota, with diet being the most critical determinant in NHPs (Gogarten *et al*. 2018; Lee *et al*. 2023; Nagpal *et al*. 2018). Our findings hold practical implications for management facilities, particularly in cage-breeding facilities, by guiding ex-situ food management protocols. Consequently, data on intestinal microbiota profiles can inform strategies for regulating nutritional intake for captive or wild primates. This could involve incorporating higher-quality natural fiber-rich foods or probiotics into the diet of captive LTM (Muhammad *et al*. 2023 ; Tian *et al*. 2022). Therefore, studies on gut microbiota profiles in NHPs, whether in wild or captive settings, are crucial for informing primate welfare management practices and will be invaluable for future management efforts.

Acknowledgements

The authors express their gratitude to the Young Lecturer Research Grant (*Penelitian Dosen Muda*) 2023, IPB University, for providing financial support for this study. Additionally, we extend our appreciation to the Primate Research Center, IPB University, and the Laboratory of Microbiology Department of Biology, IPB University for granting permission to conduct this study. RCK's effort was supported in part by the Office of Research Infrastructure Programs (ORIP) of the National Institutes of Health through grant number P51OD010425 to the Washington National Primate Research Center, USA.

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