Assessing Methodological Variability in Gut Microbiome Studies: Lessons from Southeast Asian for Effective Conservation Strategies

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Abstract. Gut microbiome studies have gained significant attention in recent years due to their potential in unveiling the role of microbial communities in animals' health and ecological processes. However, the lack of standardized protocols in sample handling and processing across studies introduces variability, impeding the comparability of findings. This study addresses this issue by examining methodological variations in gut microbiome research on wildlife and domesticated animals in Southeast Asia. A comprehensive search of 91 relevant studies on the SCOPUS database yielded 54 suitable publications for review, encompassing diverse taxa such as invertebrates (20), fishes (7), reptiles (3), birds (5), and mammals (19). Notably, various methodological approaches were employed to characterize microbial communities, including the source of isolation, various culture-based approaches, sequencing methods, and the targeted markers. Based on the information provided in this study, future studies should strive to develop guidelines and best practices specific to gut microbiome studies. This would enhance comparability and facilitate the integration of findings. Such efforts will also advance our understanding of the microbial diversity associated with wildlife, and its potential implications for their health and conservation.

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1 Introduction

Wildlife gut microbiome studies have gained substantial attention within ecological and conservation research, providing deep insights into the complex relationship between microorganisms residing in the gastrointestinal tract of various animal species. To date, the majority of microbiome studies have primarily focused on humans and domesticated animals, and for wildlife, efforts are now becoming more widespread to address this research gap [1]. This coincides with the advancement of technologies such as high-throughput sequencing which has enabled faster and cost-effective identification of microbiomes among and within wildlife [2, 3]. Generally, gut microbiome is a diverse set of microbial taxa inhabiting the gastrointestinal tract system, including their genetic material [4]. Understanding the diversity, composition, and functional roles of these microbial communities is critical for solving the complexities of ecosystems and their influence on the fitness, health, and behaviour of wildlife [5-7].

Various methodological approaches, practices, new challenges, and opportunities are emerging in the rapidly advancing field of wildlife gut microbiome research. Currently, there is no standardized set of best practices guiding the gut microbiome among wildlife. This necessitates a critical analysis of methodological and technical variability [1, 2]. Furthermore, current evidence suggests that the impacts of the gut microbiome on host species and environments differ across various aspects. These include behavior, such as social patterns and stress [8-11] and health aspects like the digestive system, hormone metabolites, and the immune system [6, 12-14]. Anthropogenic disturbances can significantly impact the gut microbiomes of wildlife, potentially rendering them more susceptible to diseases. This vulnerability arises from the fact that gut bacteria play a crucial role in the development of the mucosal innate immune system [4]. They achieve this through direct interactions with intestinal epithelial cells, acting as our first line of defense against pathogens and toxins. Hence, subsequent investigations in this area could contribute to a more effective conservation and management plan for the wildlife species, especially in cases where understanding can lead to the development of strategies and protocols to mitigate the risk of extinction for endangered species [2, 6].

However, much remains to be further explored on the wildlife species in Southeast Asian countries since the study is very scarce and limited. Despite being recognized as a global hotspot for biodiversity and endemism, this region is notably one of the most biotically threatened areas with regards to its biodiversity [15]. Therefore, this study aims to conduct a comprehensive assessment of methodological differences in gut microbiome studies, specifically focusing on studies conducted in Southeast Asian countries. Understanding this variability is crucial for accurately interpreting research findings in this region and to help provide set of protocols that minimize bias in this field, enabling better-informed conservation and management strategies tailored to the unique characteristics of our Southeast Asian wildlife.

2 Methodology

Bibliographic searches were utilized to gather data on the methodological variability in gut microbiome studies. This review consists of a few stages, which are (1) identifying the search strategy, (2) sorting the exported research articles, (3) identifying the relevant studies, and (4) summarizing and tabulating the results.

2.1 Search strategy

Peer-reviewed articles were searched for in the SCOPUS database based on indexed titles, abstracts, keywords, and topics. Several search strings were created to make sure the search was thoroughly done, combining keywords such as gut microbiome, microbes, and gastrointestinal microbiota with the names of 12 Southeast Asian countries, namely Malaysia, Indonesia, Brunei, Vietnam, Singapore, Timor Leste, Laos, Cambodia, Sri Lanka, Myanmar, Thailand, and Philippines. However, take into account that there is a possibility that potential related research articles were missed during the search and were not included in this review article.

2.2 Article selection and screening

The criteria for study inclusion were refined, where the articles were included if they met the following criteria: (a) had a focus on microbiome study (including research and reports, except review papers), (b) study on wildlife as well as domesticated/farm animals that utilized any gastrointestinal parts of the animals and (c) reported on the application for microbial identification. Only peer-reviewed research articles published in English were included. A few articles were also excluded if they were inaccessible or absence of a clear information in the study abstract or aims. Database search records were then imported for screening and listing without duplication, where in this process, full-text articles were retrieved and screened before a comprehensive Excel list of acceptable articles was developed. A complete Excel list was utilized to ease the extraction of information for each article. Data were extracted concerning the taxonomy group studied, source of isolation, identification methods used, and the targeted region. All the information gathered was then collated and summarized, and the data were presented in tables or graphs.

3 Results and Discussion

3.1 Overview of the extracted articles

Out of 36 search strings listed, results from the 29 search strings were successfully exported, while the rest did not yield related data. According to Table 1, Timor Leste is the only country not generating any related articles regarding microbial study since the keywords used showed zero results. As for Brunei, Laos, and Cambodia, the available articles in the database did not meet the inclusion criteria hence no articles could be extracted. Out of the total articles extracted, four related studies conducted in Malaysia (n=2), Indonesia (n=1), and the Philippines (n=1) were inaccessible for this review. Hence, 91 related articles were sorted and filtered; consequently, only 54 peer-reviewed articles were included in the review. Table 2 summarizes the included articles, avoiding duplication, generated from the three keywords (gut microbiome, microbes, and gastrointestinal microbiota) for each Southeast Asian country. A unique article was defined as one without any duplications; however, it's worth mentioning that duplicate articles may appear across different countries.

Table 1. List of search strings used and number of research articles in each bibliographic search.

Keywords	Number of Publication (SCOPUS)	
	Total of Research Articles Available	**Total of Extracted Articles (% Already Included in Previous Research)**
Gut microbiome AND *Malaysia*	40	9
Microbes AND *Malaysia*	191	10 (40%)
Gastrointestinal microbiota AND *Malaysia*	27	3 (66%)
Gut microbiome AND *Brunei*	0	0
Microbes AND *Brunei*	11	0
Gastrointestinal microbiota AND *Brunei*	0	0
Gut microbiome AND *Indonesia*	34	9 (11%)
Microbes AND *Indonesia*	315	7 (28%)
Gastrointestinal microbiota AND *Indonesia*	29	5 (80%)
Gut microbiome AND *Vietnam*	15	7
Microbes AND *Vietnam*	53	1 (100%)
Gastrointestinal microbiota AND *Vietnam*	13	3 (66%)
Gut microbiome AND *Singapore*	28	2
Microbes AND *Singapore*	54	4 (75%)
Gastrointestinal microbiota AND *Singapore*	16	1 (100%)
Gut microbiome AND *Timor Leste*	0	0
Microbes AND *Timor Leste*	0	0
Gastrointestinal microbiota AND *Timor Leste*	0	0
Gut microbiome AND *Laos*	0	0
Microbes AND *Laos*	8	0
Gastrointestinal microbiota AND *Laos*	1	0
Gut microbiome AND *Cambodia*	2	0
Microbes AND *Cambodia*	14	0
Gastrointestinal microbiota AND *Cambodia*	0	0
Gut microbiome AND *Sri Lanka*	5	2
Microbes AND *Sri Lanka*	22	1 (100%)
Gastrointestinal microbiota AND *Sri Lanka*	4	2 (100%)
Gut microbiome AND *Myanmar*	4	1 (100%)
Microbes AND *Myanmar*	13	1 (100%)
Gastrointestinal microbiota AND *Myanmar*	1	1
Gut microbiome AND *Thailand*	45	7 (14%)
Microbes AND *Thailand*	138	3 (66%)
Gastrointestinal microbiota AND *Thailand*	28	2 (100%)
Gut microbiome AND *Philippines*	9	4 (25%)
Microbes AND * Philippines *	40	2
Gastrointestinal microbiota AND * Philippines *	4	3 (66%)

Table 2. Total number of extracted articles and unique articles for each of the country.

Country	Total Number of Extracted Articles	Total Number of Unique Articles (No Duplication)	Suitable Peer-Reviewed Articles
Malaysia	22	16	**14**
Indonesia	21	15	**13**
Vietnam	11	8	**8**
Singapore	7	3	**3**
Sri Lanka	5	3	**3**
Myanmar	3	2	**1**
Thailand	13	9	**7**
Philippines	9	7	**5**
Brunei	0	0	**-**
Laos	0	0	**-**
Cambodia	0	0	**-**
Timor-Leste	0	0	**-**
		Total	**54**

Based on Figure 1, the data reveals a steady growth in research output over the years. The earliest related publications on gut microbiome study in Southeast Asia available in the SCOPUS database is in 2009 (n=1), where the study was conducted on fish species by Zhou et al [16] and in the following year (2010), there was also a limited publication with one research article on dogs regarding gastric disease by Camer et al [17]. These two earliest studies were conducted in the Philippines respectively. The subsequent years witnessed a gradual rise, with notable spikes in 2011 (n=2) and 2015 (n=2). The year 2018 marked a significant increase, with four research articles, demonstrating a substantial upward trajectory. Subsequently, the number of articles has increased exponentially in 2020 with 13 articles published, followed with 13 articles in 2021 and 10 articles in 2022. Even in 2023, the trend remains robust, with nine research articles already published. These numbers signify the region's increasing comprehension of the importance of microbiomes among and within animals and the host-microbiome complex relationships or roles [4, 18]. The widespread use of microbiome sequencing is also evident, given its greater feasibility and economic viability, and this development occurred in parallel with advancements in high-throughput sequencing [3, 19].

3.2 Summary of microbiome studies in Southeast Asia

Accumulating evidence from the search results suggest that most of the microbiome study in Southeast Asia focuses on humans and model organisms. Across the 54 extracted articles, generally, most of the articles covered a range of topics, with the majority focused on documenting the diversity of microbiota in the animals, including for health assessment, comparison between wild and captive groups, as well as effects of anthropogenic disturbances. Interestingly, in examining the research on the gut microbiome within Southeast Asia, a breakdown of taxonomic groups reveals that the data indicates a notable emphasis on invertebrates (including marine invertebrates), with a total of 20 studies dedicated to this taxonomic group (Figure 2). Out of the 20 studies on invertebrates, 12 of them focused on insects, particularly mosquitoes (eg. Minard et al. [20], Surat et al [21], and Rosso et al [22]) and honeybees (eg. Lombogia et al [23], Duong et al [24], Gruneck et al [25], and Lanh et al [26]), there are also a study on fleas by Rombot et al [27], wasp by

Badrulisham et al [28], termites by Simol et al [29], cockroach by Ni'matuzahroh et al [30] and weevil by Farah-Nadia et al [31]. Following closely, mammals are a prominent focus with 19 studies. Fish (*n*=7), reptiles (*n*=3) and birds (*n*=5) are also subjects of research interest, underlining the comprehensive approach to studying different animal groups.

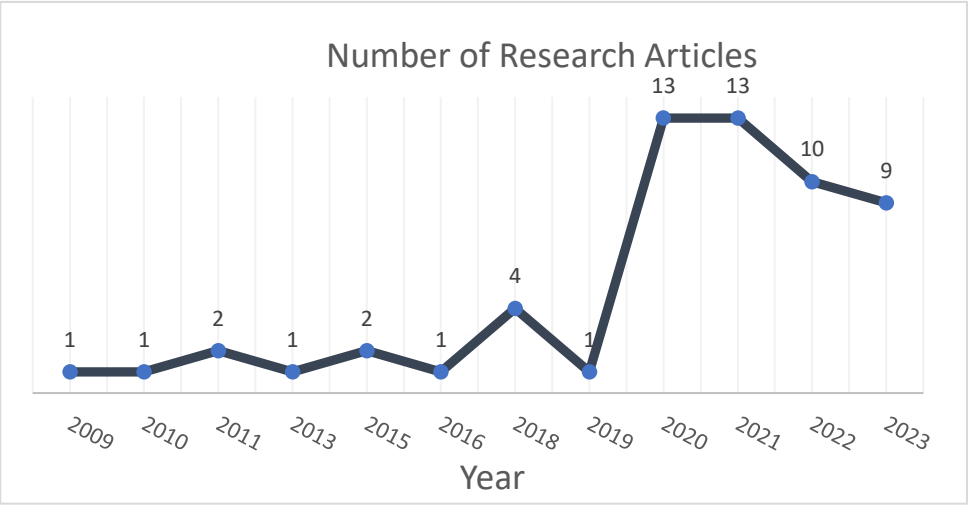

Fig. 1. Number of research articles published in Southeast Asian countries by year.

Among the conducted research, a significant proportion, comprising 76% (41 out of 54 studies) focuses on wildlife, highlighting the critical role of wildlife-microbiome research within the region. Particularly concerning conservation efforts, given the escalating fragmentation of the remaining forests in this region, it becomes imperative to prioritize investigations into host-microbiome associations for endangered species [15]. Additionally, understanding the factors facilitating the transmission of pathogens between wildlife and humans is essential in this context [6, 7]. Despite limited funding and restricted access to genomic tools in developing countries, there is anticipated growth in research in this field [3]. This is particularly notable in biodiversity monitoring, where research is expected to focus on changes at the species, population, and ecosystem levels. Conversely, 24% of studies (13 out of 54) target domesticated animals, especially those that contribute to food producing such as meat and milk, reflecting a discerning effort to comprehend the gut microbiome in both wild and domesticated perspectives. Some of the studies focused on ruminants such as cows (eg. Teo et al [32], Astriani et al [33], Hang et al [34], and Prasetiyono et al [35]) and buffaloes (eg. Harsojo [36], and Agustina et al [37]), poultries such as chickens by Pin Viso et al [38] and duck (eg. Susanti et al [39], and Susanti et al [40]), and porcines such as pigs by Ngoc et al [41].

3.3 Methodological variability in gut microbiome studies in Southeast Asia

Generally, Table 3 describes methodological variations observed in animal host-microbiome studies conducted in Southeast Asia. We categorize these studies according to their source of isolation, identification method (culture-based, sequencing or combined methods) and the selection of targeted regions when sequencing techniques are employed. As is commonly recognized, microbiome research begins by obtaining biological specimens. In this case, it typically involves both invasive and non-invasive approaches, including the collection of faecal samples, rumen fluid samples or cloacal swabs from individual hosts or

the dissection of specific regions within the host's gastrointestinal tract, such as the gut, midgut, or intestines. Our results indicate that the choice between invasive or non-invasive approaches is contingent upon the specific group of animals under study (Figure 3). Notably, all the research on invertebrates and fish employs invasive methods while non-invasive approaches are generally preferred for larger animals such as mammals. Only a limited number of studies focusing on mammals have gathered microbes directly from specific parts of the gastrointestinal tract, and these studies have primarily centred on food-producing animals such as organs of cows, pigs, and buffalo sourced from the traditional market [33, 36, 41]. This preference also can be attributed to the protected status of many wildlife species and the potential risks involved, such as the possibility of injuries or fatalities, which could have significant ethical and conservation implications, especially for endangered or threatened species [7, 15].

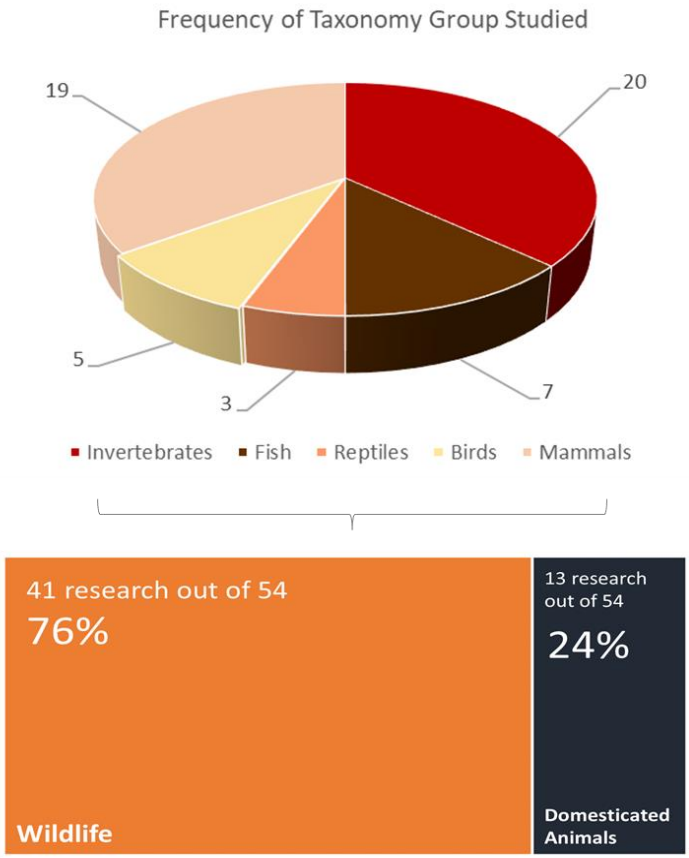

Fig. 2. Frequency of taxonomy groups studied in Southeast Asia.

Meanwhile, non-invasive sampling methods, like the collection of faecal samples, offer practical advantages by allowing researchers to sample individuals repeatedly over time. However, it is important to note that faecal samples can be susceptible to contamination, potentially compromising the consistency of microbial data if they are not collected promptly [42]. In wildlife studies, the difficulties in tracking many wildlife species add complexity because the precise timing of faecal sample deposition is uncertain and eventually the exposure can alter the original microbial communities in the sample [43]. Overall, non-

invasive approaches have witnessed significant advancements in genetics research [44]. This is because DNA can be extracted from sources such as faeces, shed hairs or feathers, saliva, and other materials. In contrast, invasive methods often require the physical capture or handling of animals, which can lead to significant stress, injuries, or even mortality. Stress-induced reactions can lead to data distortion, compromising the study's reliability of results [42]. But when considering microbial richness and diversity based on the source of biological specimens, it is worth noting that direct gastrointestinal samples may provide a more representative insight into the endogenous microbiome within a host [45-47]. This can be substantiated by research conducted by Durbán et al [45], which confirms that the intestinal microbiota is an exceptionally complex community where its richness and diversity appear to be underrepresented in faecal samples. However, it is essential to acknowledge that direct comparisons of the results gained between different sampling methods may be challenging due to the multitude of varied factors influencing these results. In summary, each collection and sampling method has its practical advantages and limitations that need thorough consideration when choosing the most suitable approach. Furthermore, each sample type can offer a unique and complementary perspective on the diversity and ecology within the hosts' gut microbiota.

	Invertebrates/ Marine Invertebrates	Fish	Reptiles	Birds	Mammals
Dissection of Gastrointestinal Tract	20	7		2	4
Cloacal/Rectum Swab			2	1	2
Faecal Sample			1	3	12
Gastric/Rumen Fluid					3

Fig. 3. Heatmap for source of isolation based on the taxonomy group of the host.

Following sample collection, the microbial identification or characterization process is typically initiated. Based on the assessment, it is evident that the majority of research in microbiome studies has transitioned from primarily culture-based approaches, which focused on individual species within an ecosystem, to the comprehensive analysis of entire microbial communities, facilitated by the advancements in sequencing technologies and eliminating the need for species cultivation. Table 3 exhibits extracted information from articles that utilized culture-based approaches for microbial identification. 12 articles were extracted, with the articles listed in the table dating from 2011 until 2023. Among the earliest studies on animal microbiomes were those focused on domesticated production animals, such as cows [32, 48]. According to Peixoto et al [49], studying the rumen microbiome is crucial as it affects the nutrition, health, animal products, by-products, and will indirectly affecting the human health from consumption.

Out of the 12 studies that employed the conventional approaches, half of them utilized morphological and biochemical examinations for the microbial identification process, while the other half employed the polymerase chain reaction (PCR) for identification. The latter is sometimes referred to as the "polyphasic" approach, characterized by its emphasis on integrating morphological and biochemical data with molecular techniques [50]. Typically, research aiming to identify specific targeted bacterial species utilizes culture-based methods, incorporating morphological criteria such as colony shape, color, size, Gram staining, and

biochemical characteristics for the identification process. For example, Suharti et al [51] used Carboxy Methyl Cellulose (CMC) media to detect the presence of cellulolytic bacteria in the gastrointestinal tracts of tropical herbivores. An additional instance, Ni'matuzahroh et al [30] studied cockroach midgut endosymbiont bacteria by cultivating them on nutrient agar medium and then subjected the samples to microscopic observation for Gram staining, analysis of bacterial cell morphology, and employed a kit to identify potential endosymbiotic microbes. Despite that, these conventional approaches are time consuming and require labour intensive especially in culturing media and biochemical tests [50, 52]. But Hameed et al. [53] suggested that combining conventional and molecular techniques can help achieve the accuracy, rapidness, and reliability of microbiome results. Hence, the technique also been proven in a few groups of wildlife studies throughout Southeast Asia [23, 29, 33, 51, 54, 55].

The introduction of sequencing technology has significantly accelerated the discovery of microbiomes, including the identification of pathogens, in a much shorter time [52]. Table 4 provides a summary of the information extracted from each article using sequencing technology approaches. We can observe a transition from DNA-based (Sanger) methods to short-read sequencing with platforms like Roche 454, Illumina and BGISEQ-500 technologies, and long-read technologies like Nanopore (Figure 4). According to the articles extracted in Southeast Asia in 2009 and 2013, Sanger sequencing was the primary method of choice, with one study utilizing this technology. Subsequently, in 2015, researchers began to adopt Illumina sequencing, marking a shift towards next-generation sequencing methods. Moving forward, the sequencing landscape continued to diversify, and the number of research studies conducted on this topic increased (refer to Figure 1), demonstrating the ongoing advancements in sequencing technology and better accessibility to newer platforms to meet the evolving demands of the latest research. One notable aspect of these advancements is the reduced time required to obtain results when utilizing newer platforms [3]. However, in long-read sequencing and shotgun metagenomics, PCR-free metagenomic analysis or shotgun sequencing usually require a substantial amount of initial DNA, thus using a low quantity of input DNA can result in reduced sequencing data output, which, in turn, may impact the accuracy of microbial community composition determination [56]. Generally, the selection of technology depends on multiple factors, such as sample type the desired depth and accuracy of results, available resources and cost considerations, as well as the expected duration of completing times [50]. These factors influence the advantages and limitations associated with each approach.

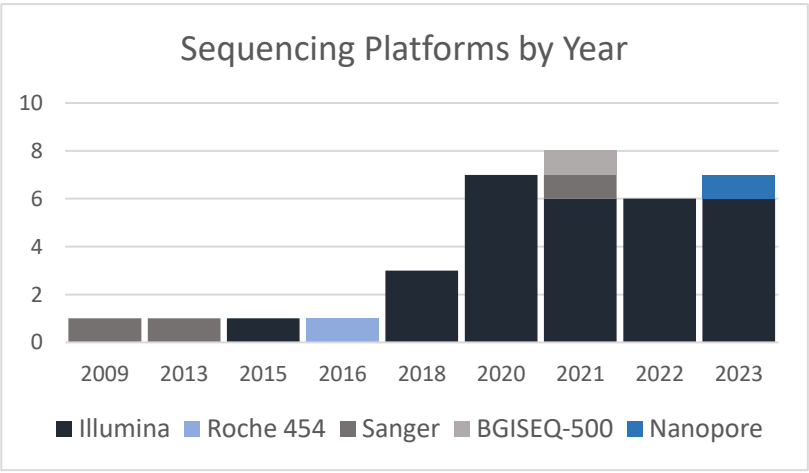

Fig. 4. Sequencing platforms trend over the years.

Table 3. Summary of the extracted information from each article from Southeast Asian countries using culture-based approaches.

Country	Group	Study Species	Source of Isolation	Identification Platform	Target Regions for DNA Extraction	Author
Malaysia	Invertebrates/Marine Invertebrates	Termite (*Coptotermes curvignathus*)	Gut	Culture Media-PCR	16s rRNA	Simol et al [29]
		Sea Cucumber *(Holothuria leucospilota)*, Gamat (*Stichopus horrens)*	Intestine	Culture Media-PCR	16s rRNA	Kamarudin et al [54]
	Mammals	Cow	Faecal sample	Culture Media	N/A	Teo et al [32]
Indonesia	Invertebrates/Marine Invertebrates	Honeybee (*Apis nigrocincta*)	Gut	Culture Media-PCR	16s rRNA	Lombogia et al [23]
		Surinam Cockcroach *(Pynocelus surinamensis)*	Gut	Culture Media	N/A	Ni'matuzahroh et al [30]
	Mammals	Anoa *(Bubalus depressicornis)*, Banteng *(Bos javanicus)*, Muntjak *(Muntiacus muntjak)*, Timor Deer *(Rusa timorensis)*	Faecal sample	Culture Media-PCR	16s rRNA	Suharti et al [51]
		Buffalo	Rumen Fluid	Culture Media	N/A	Agustina et al [37]
		Cow	Rumen	Culture Media-PCR	16s rRNA	Astriani et al [33]
		Buffalo	Bowel	Culture Media	N/A	Harsojo [36]
Vietnam	Fish	Freshwater fish	Gut	Culture Media-PCR	ESBL genes	Hoa et al [55]
Thailand	Mammals	Cow	Faecal sample, Rumen fluid	Culture Media	N/A	Wanapat et al [48]
Philippines	Invertebrates/Marine Invertebrates	Abalone *(Haliotis asinine)*	Gastrointestinal tract	Culture Media	N/A	Mabuhay-Omar et al [57]

*N/A- Not applicable as morphological and biochemical examination were applied for identification process.

Table 4. Summary of the extracted information from each article from Southeast Asian countries using sequencing technology approaches.

Country	Group	Study Species	Source of Isolation	Sequencing Platform	Target Regions	Author
Malaysia	Invertebrates/Marine Invertebrates	Sea Cucumber *(Stichopus ocellatus)*	Gut	Illumina iSeq	16s rRNA (V4)	Choo et al [58]
		Sea Cucumber *(Stichopus ocellatus)*	Gut	Not Stated	16s rRNA (V3)	Wei et al [59]
		Parasitic Wasp (*Dolichogenidea metesae*)	Gut	Illumina Miniseq	16s rRNA (V3 and V4)	Badrulisham et al [28]
		Red Palm Weevil (*Rhynchophorus ferrugineus*)	Gut	Illumina HiSeq	16s rRNA (V1 and V2)	Farah-Nadiah et al [31]
	Fish	Fish	Gut	Illumina Miseq	16s rRNA (V3 and V4)	Saadu et al [60]
	Reptiles	Southern River Terrapin (*Batagur affinis*)	Faecal sample	Illumina Miseq	16s rRNA (V3 and V4)	Salleh et al [61]
	Birds	Chicken	Faecal sample	Illumina Miseq	16s rRNA (V3 and V4)	Pin Viso et al [38]
	Mammals	Malayan tiger *(Panthera tigris jacksoni)*	Faecal sample	Illumina Miseq	16s rRNA (V3 and V4)	Khairulmunir et al [62]
		Island Flying Fox *(Pteropus hypomelanus)*	Faecal sample	Illumina Miniseq	16s rRNA (V3 and V4)	Mohd-Yusof et al [63]
		Long-tailed macaque (*Macaca fascicularis*), Silvered leaf monkey (*Trachypithecus cristatus*)	Faecal sample	Illumina Miseq	16s rRNA	Chong et al [64]
		Short-nosed fruit bat *(Cynopterus brachyotis)*	Gut, Intestine	Sanger sequencing	16s rRNA	Daniel et al [65]
Indonesia	Invertebrates/Marine Invertebrates	Ticks *(Rhipicephalus sanguineus)*	Gastrointestinal tract	Not Stated	16s rRNA (V3 and V4)	Tahulending et al [66]
	Fish	Zebrafish (*Danio rerio*)	Gut	Illumina Miseq	16s rRNA (V3 and V4)	Chen et al [67]
	Birds	Duck	Faecal sample, Intestine	Illumina Miseq	16s rRNA (V3 and V4)	Susanti et al [39]
		Sulawesi Babbler (*Pellorneum celebense*)	Cloacal	Illumina Miseq	16s rRNA	Joakim et al [68]
		Duck *(Anas platyrhynchos)*	Intestine	Illumina HiSeq	16s rRNA (V3 and V4)	Susanti et al [40]
	Mammals	Indo-Pacific Bottlenose Dolphin (*Tursiops aduncus*)	Faecal sample, Gastric fluid	Illumina	16s rRNA (V3 and V4)	Indrawati et al [69]
		Cow	Faecal sample	Illumina	Not Stated	Prasetiyono et al [35]
Vietnam	Invertebrates/Marine Invertebrates	Honeybee *(Apis cerana)*	Gut	Illumina MiSeq	16s rRNA (V3 and V4)	Lanh et al [26]
		Honeybee *(Apis cerana)*	Gut	Illumina MiSeq	16s rRNA (V3 and V4)	Duong et al [24]
		Mosquitoes *(Aedes albopictus, Aedes koreicus)*	Midgut	Illumina MiSeq	16s rRNA (V5 and V6)	Rosso et al [22]

		Asian tiger mosquito *(Aedes albopictus)*	Midgut	Illumina MiSeq	16s rRNA (V5 and V6)	Minard et al [20]
		Rabbit fish *(Siganus guttatus)*	Gut	Illumina MiSeq	16s rRNA	Le et al [70]
	Mammals	Pig	Gut, Ileum, Colon	Sanger sequencing	16s rRNA (V3)	Ngoc et al [41]
		Cow	Faecal sample	Illumina HiSeq	16s rRNA (V4)	Hang et al [34]
Singapore	Invertebrates/Marine Invertebrates	Cat flea *(Ctenocephalides felisis)*	Gastrointestinal tract	Not Stated	16s rRNA (V3 and V4)	Rombot et al [27]
	Fish	Asian seabass *(Lates calcarifer)*	Gut, Intestine	Illumina Novaseq	16s rRNA (V3 and V4)	Chew et al [71]
	Mammals	Asian langurs	Faecal sample	Illumina MiSeq, Illumina HiSeq	16s rRNA	Hale et al [72]
Sri Lanka	Invertebrates/Marine Invertebrates	Mosquitoes *(Culex tritaeniorhynchus, Culex gelidus, Mansonia annulifera)*	Midgut	Not Stated	16s rRNA	Gunathilaka et al [73]
		Mosquitoes *(Aedes aegypti, Aedes albopictus)*	Midgut	Not Stated	16s rRNA	Ranasinghe et al [74]
	Mammals	Tufted gray langur *(Semnopithecus priam)*, Purple-faced langur *(Semnopithecus vetulus)*	Faecal sample	Illumina MiSeq	16s rRNA (V4)	Amato et al [75]
Myanmar	Invertebrates/Marine Invertebrates	Earthworm	Intestine	Illumina MiSeq	16s rRNA (V3 and V4)	Mathipi et al [76]
Thailand	Invertebrates/Marine Invertebrates	Dwarf Honeybee *(Apis florea)*, Giant Honeybee *(Apis dorsata)*	Gut	Not Stated	16s rRNA	Gruneck et al [25]
		Mosquito *(Anopheles minimus)*	Gut	GS-FLX Titanium	16s rRNA	Surat et al [21]
	Fish	Freshwater ricefish *(Oryzias minutillus)*, Brackish water ricefish, *(Oryzias javanicus)*	Gut	BGISEQ-500	16s rRNA (V1-V3)	Ngamniyom et al [77]
	Mammals	Long-tailed macaque (*Macaca fascicularis*)	Rectum Swab	MinION Mk1C	16s rRNA (V1-V9)	Sawaswong et al [78]
		Long-tailed macaque (*Macaca fascicularis*)	Rectum Swab	Illumina MiSeq	16s rRNA	Sawaswong et al [79]
		Long-tailed macaque (*Macaca fascicularis*)	Faecal sample	Illumina MiSeq	ITS2 gene	Sawaswong et al [80]
Philippines	Fish	Yellow grouper *(Epinephelus awoara)*	Gastrointestinal tract	Sanger sequencing	16s rRNA (V3)	Zhou et al [16]
	Birds	Duck	Faecal sample	Not Stated	16s rRNA	Rosa et al [81]
	Reptiles	Geckos	Cloacal Swab	Illumina MiSeq	16s rRNA (V4)	Eliades et al [82]
		Blue-lipped sea krait *(Laticauda laticaudata)*, Philippine pit viper *(Trimeresurus flavomaculatus)*, Mangrove snake *(Boiga dendrophila)*	Cloacal Swab	Illumina MiSeq	16s rRNA (V4)	Smith et al [83]

Regarding the choice of gene for region selection, a majority of studies (*n*=45, 83%) have employed the 16s rRNA gene in their sequencing methods, often regarded as a gold standard for bacterial identification (Figure 5) [84]. This versatility makes it an ideal choice for application in laboratory and clinical settings, including the investigation of microbiomes in different wildlife localities. For instance, Mohd-Yusof et al [63] utilized this gene to analyze the gut microbiome composition in flying foxes across various island populations on Peninsula Malaysia's east and west coasts. However, certain challenges persist when utilizing the 16s rRNA gene, primarily owing to its relatively low taxonomical resolution, which can pose difficulties in distinguishing among all bacterial taxa [85]. Nevertheless, this challenge can be addressed, as the 16S rRNA gene, with approximately 1600 base pairs long contains nine hypervariable regions (V1 until V9).

As Baker et al [86] highlighted, these regions are valuable for sequencing as they offer detailed insights into the differentiation of closely related microbial taxa. Furthermore, the highly conserved sequences surrounding these hypervariable regions serve as effective PCR priming sites for amplifying 16s genes across a diverse range of higher-ranking taxa, whereas more quickly evolving ones can help identify genus or between the species [87]. Our study reveals a notable preference in Southeast Asian gut microbiome research for the utilization of the V3-V4 regions. This inclination may be attributed to the widespread use of Illumina sequencing platforms, as the official Illumina protocol endorses the V3–V4 region. Consequently, these two regions have become widely adopted in gut microbiota studies in this region. Having said that, Bukin et al [88] assert that, contrary to using the V3-V4 regions, employing the V2-V3 regions in conjunction with the Illumina MiSeq platform provides a more precise depiction of lower-rank taxa resolutions (genus and species). But the issue of lower resolution at the species level associated with V3-V4 fragments can be addressed by adjusting the threshold for genetic distances utilized in OTU clustering.

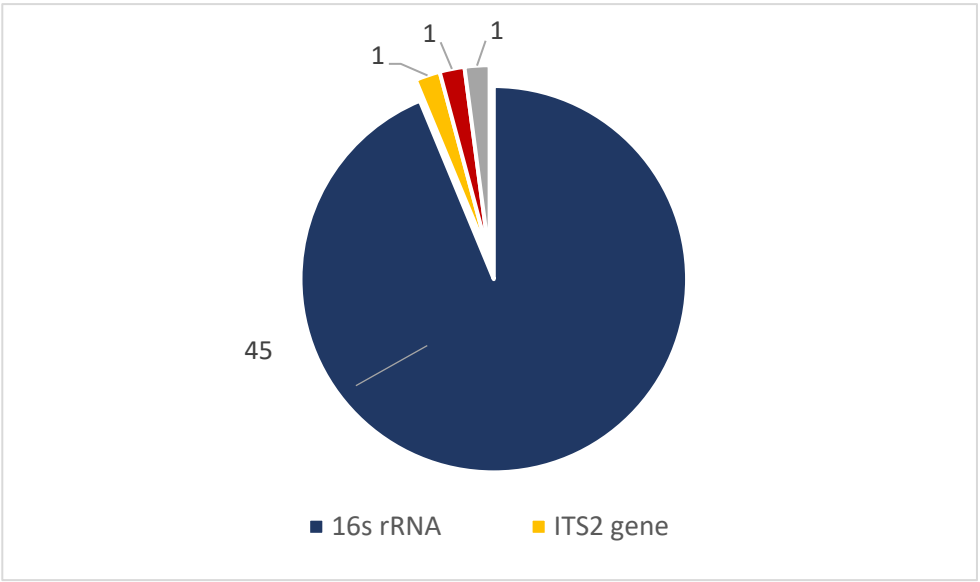

Fig. 5. Preference of targeted markers in gut microbiome studies around Southeast Asia region

However, the marker preference trend is not limited solely to this gene. In some instances, researchers have explored alternative markers, albeit less frequently. For instance,

the Internal Transcribed Spacer 2 (ITS2) gene has been employed in one study on long-tailed macaque [80], while another study on freshwater fish [55] ventured into investigating the Extended-Spectrum Beta-Lactamase (ESBL) gene. These divergent choices indicate a growing curiosity within the scientific community to explore the potential of alternative markers for more specialized research questions. Overall, no specific region can be universally regarded as a standard marker. The selection depends on the types of taxa involved, the platforms used, and even the specific target variable region of the 16s rRNA gene. Eventually, this choice will significantly impact the analysis results [89, 90].

4 Conclusion

In conclusion, assessing methodological variability in animal microbiome studies across Southeast Asia has illuminated critical insights that hold significance for developing effective conservation strategies. This evaluation has revealed a dynamic landscape, witnessing a transformation in research techniques, from traditional culture-based methods to the modern high-throughput sequencing approaches. Moreover, the choice of sequencing platforms, such as Illumina, Roche 454, BGISEQ-500, and Nanopore, reflects researchers' relentless pursuit of cutting-edge technologies to meet the evolving demands of their studies. Our analysis has also highlighted the indispensable role of the various gene markers, especially the 16s rRNA gene in microbiome investigations. This research sheds light on the methodological disparities in current animal microbiome studies and provides a foundation for standardizing methodologies within the field. Ultimately, this analysis strives to equip researchers with the knowledge necessary to enhance the effectiveness of conservation strategies and increasing our ability to monitor and mitigate zoonotic disease outbreaks, as one of the efforts to ensure the long-term well-being of our wildlife populations.

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